Fall 12-1-2019

Determining the Role of TLR2 and TLR3 Synergy from Myd88-Dependent Inflammatory Responses

Hellen Teixeira

Determining the Role of TLR2 and TLR3 Synergy from Myd88-Dependent Inflammatory Responses

Abstract

The function of the adaptor molecule MyD88 is thought to be independent of toll-like receptor 3 (TLR3) signaling. This study aimed to identify certain previously unknown roles of MyD88 in TLR3 signaling during the promotion of pro-inflammatory cytokine production. Upon conducting an analysis of all the TLR ligands, it was found that the TLR3-specific ligand polyinosinic: polycytidylic acid (Poly I:C) significantly induced the production of TNF proteins and up-regulation of other TLR transcripts, particularly TLR2. Accordingly, TLR3 stimulation also led to a significant up-regulation of the endogenous TLR2 ligands HMGB1 and Hsp60. In contrast, *TLR3* silencing significantly down-regulated MyD88 and TLR2 expression, and pro-inflammatory IL-1β, TNF, and IL-8 cytokine secretion. The silencing of *MyD88* similarly led to the down-regulation of TLR2, IL1β, TNF, and IL-8, which suggests that MyD88 was active downstream of TLR3. The animal model, i.e., the MyD88 knockout mouse presented with lower TNF, NF-κB , and IRF-3 levels, as compared to those in the control wild type mouse treated with Poly I:C. Taken together, our results demonstrate a previously unknown role of MyD88 in the TLR3 signaling pathway; this finding highlights the importance of TLRs and adapter protein interplay for the modulation of the endogenous TLR ligands involved in pro-inflammatory cytokine regulation.

Degree Type

Thesis

Degree Name

DScD (Doctor of Science in Dentistry)

Primary Advisor

Dr. Dana Graves

Keywords

Toll-like receptors, innate immune system, immunology, Poly I:C

Subject Categories

Dentistry

UNIVERSITY OF PENNSYLVANIA

SCHOOL OF DENTAL MEDICINE

Determining the role of TLR2 and TLR3 synergy from Myd88-dependent inflammatory responses

Hellen Teixeira

Submitted in partial fulfillment of the Requirements for the Degree of Doctor of Science in Dentistry

2019

Dissertation Supervisor:

Dr. **Dana T. Graves**, DDS, DMSc

Professor, Vice Dean for scholarship and research.

Director, Doctor of Science in Dentistry program.

Department of Periodontics.

University of Pennsylvania School of Dental Medicine.

Dissertation Co- supervisor:

Dr. **Manjunatha Benakanakere**, PhD

Faculty, Department of Periodontics.

University of Pennsylvania School of Dental Medicine.

Chairperson of Dissertation Committee:

Dr. **Hyun** (Michel) **Koo,** DDS, MS, PhD.

Professor in the Department of Orthodontics, Pediatric Dentistry & Community Oral Health Divisions.

University of Pennsylvania School of Dental Medicine.

Dissertation Committee:

Dr. **George Hajishengallis,** DDS, PhD.

Thomas W. Evans Centennial Professor, Department of Microbiology.

University of Pennsylvania School of Dental Medicine

Dr. **Claire Mitchell**, PhD

Professor at the Department of Physiology.

University of Pennsylvania School of Dental Medicine

ACKNOWLEDGMENTS

I would like to thank my faculty, professors, colleagues, friends, and family for their guidance and support. I would especially like to thank and acknowledge my supervisor Dr. Dana Graves, for providing me with endless support and guidance. I am grateful to Dr. Manjunatha Benakanakere, for his support, guidance, and help during the development and execution of this project.

I wish to express my gratitude to the faculty of the Orthodontic Department, specially Dr. Chun-Hsi Chung, Dr. Robert Vanarsdall, Dr. Jerome Sclaroff, Dr. Peter Greco, and Dr. Guy Coby, for providing their support and guidance during my 5-year-long incredible and educational journey.

I would also like to thank my committee members, i.e. Dr. Michel Koo, Dr. George Hajishengallis, and Dr. Claire Mitchel. Their expertise and vast experience in mentoring doctorate students inspired me to successfully perform advanced scientific research, and attempt to achieve my highest educational degree. I also wish to thank Dr. Kinane and his team, especially Livia Finoti, Hanadi Alyami, and Daniela Palioto Bulle. Thank you for sharing your precious knowledge and time for training me with regard to all the laboratorial techniques used in our lab.

Most importantly, I would like to thank my family and friends, as I could not have achieved my incredible and extensive academic education without their support, care, and love.

ABSTRACT

The function of the adaptor molecule MyD88 is thought to be independent of toll-like receptor 3 (TLR3) signaling. This study aimed to identify certain previously unknown roles of MyD88 in TLR3 signaling during the promotion of pro-inflammatory cytokine production. Upon conducting an analysis of all the TLR ligands, it was found that the TLR3-specific ligand polyinosinic: polycytidylic acid (Poly I:C) significantly induced the production of TNF proteins and up-regulation of other TLR transcripts, particularly TLR2. Accordingly, TLR3 stimulation also led to a significant up-regulation of the endogenous TLR2 ligands HMGB1 and Hsp60. In contrast, *TLR3* silencing significantly down-regulated MyD88 and TLR2 expression, and pro-inflammatory IL-1β, TNF, and IL-8 cytokine secretion. The silencing of *MyD88* similarly led to the down-regulation of TLR2, IL1β, TNF, and IL-8, which suggests that MyD88 was active downstream of TLR3. The animal model, i.e., the MyD88 knockout mouse presented with lower TNF, NF-κB , and IRF-3 levels, as compared to those in the control wild type mouse treated with Poly I:C. Taken together, our results demonstrate a previously unknown role of MyD88 in the TLR3 signaling pathway; this finding highlights the importance of TLRs and adapter protein interplay for the modulation of the endogenous TLR ligands involved in pro-inflammatory cytokine regulation.

TABLE OF CONTENTS

Table of Contents

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

ANOVA: Analysis of variance

cDNA: Complementary DNA

DMSO: Di-methylsulfoxide

DNase: Deoxyribonuclease

ELISA: Enzyme-linked immunosorbent assay

FBS: Fetal bovine serum

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GPCR: G-protein-coupled receptors

sgRNA: Single guide ribonucleic acid

HS: Horse serum

Hsp60: Heat shock protein 60

HMGB1: High mobility group protein B1

IL-8: Interleukin 8

LPS: Lipopolysaccharide

NFκB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NOD: Nucleotide-binding oligomerization domain

PAM: Protospacer adjacent motif

PAMPs: Pathogen-associated molecular patterns

PBS: Phosphate buffered saline

PMA: Phorbol myristate acetate

PMNL: Polymorphonuclear leukocytes

PRRs: Pattern-recognition receptors

PYD: Pyrin domain

qPCR: Quantitative polymerase chain reaction

RIPA: Radioimmunoprecipitation assay buffer

RNase: Ribonuclease

ROS: Reactive oxygen species

RT-PCR: Real time polymerase chain reaction

TLRs: Toll-like receptors

Chapter 1: Introduction

The molecular adaptor Myd88 participates in the inflammatory pathways of most Toll-like receptors (TLRs) (McNutt 1974, Benakanakere, Li et al. 2009, Zhao, Benakanakere et al. 2010, Kennedy, Najdovska et al. 2014). However, the roles of Myd88 in the TLR3 pathway and the synergistic interactions between TLR3 and TLR2 have never been investigated (Teixeira, Zhao et al. 2019). Epithelial cells are known to be the first line of host defense against invading pathogens. TLRs play a key role in the recognition of either bacterial or viral pathogens and the subsequent activation of innate immune responses, for establishing homeostasis during an infection (Brown, Wang et al. 2011, Xia, Winkelmann et al. 2013, Qin, Li et al. 2016). Under homoeostasis, an array of these pattern-recognition receptors (PRRs) in the immune system detect pathogen-associated molecular patterns (PAMPs) and trigger an effective immune response (Koticha 1969, Du, Liu et al. 2018). In individuals with dysbiosis, the overactivation of these TLRs will lead to chronic inflammation. The TLRs are classified based on their localization (intracellular or extracellular) and corresponding ligands. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 recognize microbial membrane components, such as lipoproteins, and are normally expressed on cell surfaces. Conversely, TLR3, TLR7, TLR8, and TLR9 detect pathogenic nucleic acids and are found intracellularly in vesicles, including endosomes, those of the endoplasmic reticulum (ER), endolysosomes, and lysosomes (Koticha 1969, Akira, Yamamoto et al. 2003).

Due to the large variety of microorganisms in biofilms, the oral mucosal surface is continuously exposed to commensals and pathogens. In chronic oral infections, certain types of pathogens might induce an unrestrained immune response that affects oral

immune homeostasis. In individuals with periodontal infections, human gingival epithelial cells (HGECs) play a crucial role in maintaining the homeostasis of the oral innate immune system, by protecting against bacterial insult via TLR activation. The event is characterized by an increase in pro-inflammatory cytokine and antimicrobial peptide production in response to bacterial disruption (Barton and Medzhitov 2002, Getz 2005, Kawai and Akira 2010). Various types of TLRs are expressed by HGECs, which specifically recognize TLR2 and TLR4 of *Porphyromonas gingivalis*, a well-known gram negative bacterial pathogen. Hence, TLR2 is considered an important receptor for *P. gingivalis*-related changes in sensation and innate immune response mediation. TLR2 can detect a wide range of pathogens, such as fungi, bacteria, viruses, and parasites. TLR2 is involved in the recognition of Gram positive bacterial peptidoglycans, lipoteichoic acid, fungal zymosan, *mycobacterial* lipoarabinomannan, viral hemagglutinin protein, *Trypanosoma cruzi* tGPI-mucin, and bacterial lipopeptides. To detect triacylated lipopeptides in mycoplasma and Gram negative bacteria, TLR2 forms heterodimers with TLR1; for the detection of diacylated lipopeptides in Gram positive bacteria and mycoplasma, TLR2 forms a heterodimer with TLR6. Moreover, TLR2 works in collaboration with other cell surface co-receptors (Ding, Liu et al. 2015). It was believed that the production of inflammatory cytokines, and not type I interferons, can be induced by TLR2 agonists in macrophages and dendritic cells. On the other hand, studies have shown that TLR2 agonists enable the generation of type I interferons by inflammatory monocytes in response to vaccinia virual infections (Akira and Takeda 2004, Latorre, Mendoza et al. 2014).

While TLR2 plays an important role in HGECs, we and others have shown that epithelial cells also express high levels of TLR3, as compared to the expression levels of other TLRs. We have shown that in HEGECs, TLR3 can induce robust inflammatory cytokine generation through the activation of the mTOR signaling pathway. TLR3 recognizes a synthetic double-stranded RNA (dsRNA) analog, polyinosinic-polycytidylic acid (Poly(I:C)), and promotes the production of both inflammatory cytokines and type I interferons as an antiviral response, thus serving an important role in preventing virual infections (Zhao, Benakanakere et al. 2010).

Toll/interleukin-1 (TIR) is an intracellular domain found on an adaptor molecule named myeloid differentiation primary response 88 (MyD88) (Gray, Dunne et al. 2006). Its discovery led to intense studies of TLR signaling pathways. The importance of the TIR domain in the selective recruitment of distinct adaptor molecules by TLRs was emphasized by the identification of additional TIR domain-containing adaptors. This selective recruitment results in more specific immunological responses being directed against the infecting pathogen. The activation of the transcription factor NF-κB and mitogen-activated protein kinases (MAPKs), which stimulate the generation of inflammatory cytokines in all TLRs except for TLR3, is mediated by MyD88, the first identified member of this TIR family. In contrast, the activation of transcription factors such as IRF3 and NF-κB and the subsequent activation of type I interferons and inflammatory cytokines is mediated by TIR-domain-containing adapter-inducing interferon-β (TRIF) via TLR3 and TLR4 stimulation (Thorburn, Tseng et al. 2016). Studies have shown that MyD88 plays no role in the activation of TLR3 pathways; however, the

expression of MyD88 with regard to the interaction of TLR2 and TLR3 has never been illustrated. After identifying TLR3 as one of the important receptors in the HGEC inflammatory cytokine network (Zhao, Benakanakere et al. 2010, Teixeira, Zhao et al. 2019), we hypothesized that it might control the expression of other TLRs and might participate in Myd88-dependent pro-inflammatory cytokine secretion via the expression of endogenous TLR ligands, as observed in other systems, via downstream signaling.

Innate Immune System:

The host defense system is composed of adaptive and innate immune responses. The innate immune system is responsible for providing the first line of defense against infections; on the other hand, the adaptive immune system causes the generation of more specific responses at a later period, as a defense mechanism, to eliminate these infections. The innate immune responses lack specificity and are encountered in healthy hosts, and serve o rapidly eliminate invading pathogens. Adaptive immune responses can be either specific or acquired immune responses. Adaptive immune responses represent a more specific type of immune defense, which is triggered in response to invading microbes (McNutt 1974, Melvold and Sticca 2007, Kennedy 2010).

Epithelial cells are considered as barriers, and represent the first line of defense in innate immunity. These cells are specialized and produce the natural antibiotics encountered in the epithelia; as they block the entry of all microbes, they are considered to be the first line of defense for the inhibition of further contact and penetration in the host. Micro-organisms that are able to enter the epithelial barrier and penetrate the tissues or blood

system are eliminated by other innate immune defense mechanisms such as phagocytes or nets formed by neutrophils (Jillson 1982, Parham 2006). Natural killer (NK) cells and plasma proteins, such as the proteins of the complement system also participate in the elimination of the micro-organisms that attempt to enter the host environment. Usually, different components of the innate immune system react only to harmful foreign products and specific molecules produced by different types of microbes, viruses, or bacteria. Despite this, innate immune responses contribute to the enhancement of adaptive immune responses against microbes. Innate immunity is responsible for providing a preliminary defense against infections (Parham 2006). This preliminary defense mechanism prevents the occurrence of infections via the use of epithelial barriers or by the killing of microbes (e.g., phagocytes, NK cells, the complement system). Adaptive immunity is partially controlled by lymphocytes and their products. An antibody response leads to the prevention of infections and eradication of microbes, while T lymphocytes can eliminate intracellular microbes (Jillson 1982, Parham 2006).

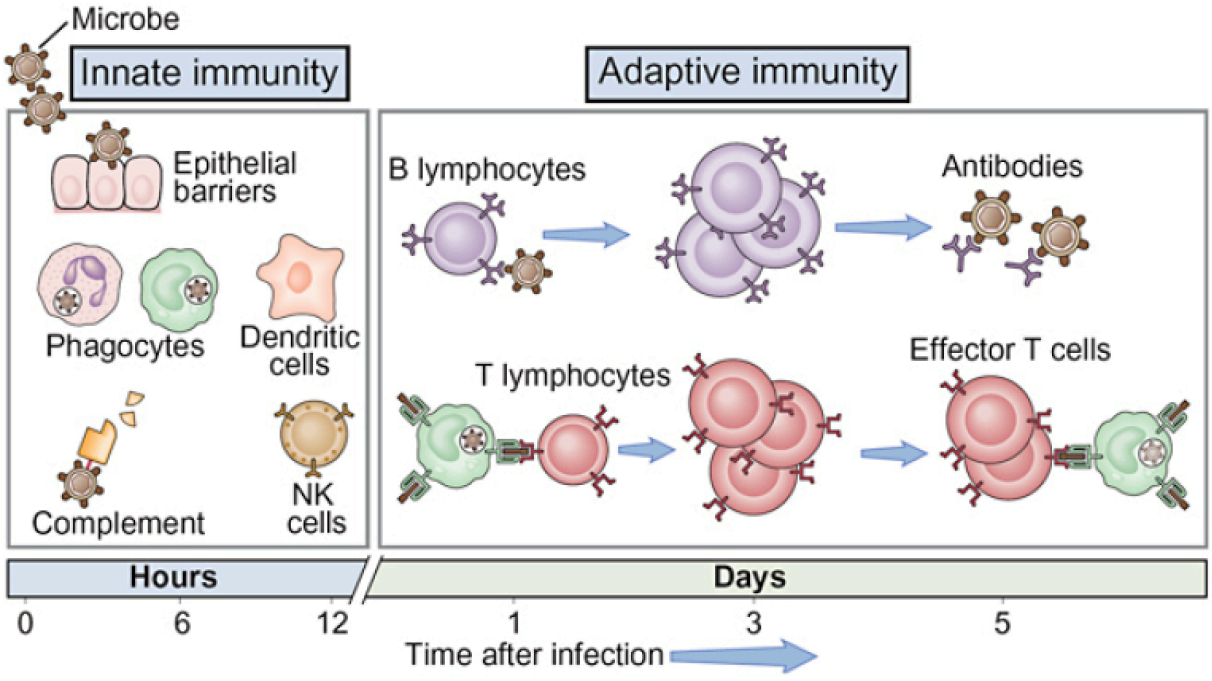

Figure 1 : **Innate immune system and the initial defense against infections**:

Initial responses prevent penetration by invaders (e.g., epithelial barriers) and eliminate microbes (e.g., phagocytes, natural killer [NK] cells, and the complement system). Adaptive immune responses develop later and are controlled by lymphocytes and their products. Antibodies block infections and T lymphocytes eliminate pathogenic microorganisms (McNutt 1974).

The principal barriers between the host and the environment include the epithelia of the skin, and the gastrointestinal and respiratory tracts. Epithelia create physical and functional barriers against harmful microbes that attempt to cross over beyond the epithelial cells and colonize the host (McNutt 1974, Jillson 1982, Parham 2006). The epithelia not only provide the physical and functional barriers, but are also responsible for the production of antimicrobial agents that reduce the grown of certain microbes.

Neutrophils and macrophages are able to ingest microbes into phagosomes, which are vesicles filled with enzymes that are able to destroy the microbes. Microbicidal substances are produced in these phagosomes. Neutrophils also have a secondary host mechanism of defense called net formation. Neutrophils form a net in which microbes are trapped and engulfed. Macrophages and dendritic cells have the ability to produce cytokines; these soluble proteins can potentially induce inflammation and trigger lymphocyte responses. NK cells are able to produce the macrophage-activating cytokine interferon-γ (IFN-γ), which can kill cells infected with viruses (McNutt 1974). The compliment system and plasma proteins can be triggered by bacteria and viruses. The activation of the compliment system or cytokines leads to the formation of products that would eliminate microbes and cause signal generation by coating their surfaces, to enable their phagocytosis by neutrophils and macrophages. The most fascinating function of the innate immune system is not only the eradication of some infections, but the identification of other microbes that need to be destroyed by the adaptive immune system (McNutt 1974, Jillson 1982).

Innate Immune System: TLRs

The most significant problem in immunology is to clarify how a host organism can become aware of and identify the presence of infectious bacteria, viruses, and other microbes, and eradicate the foreign body without destroying its own tissues (McNutt 1974, Akira and Takeda 2004). This is a significant problem, because of the huge molecular diversity of microbes and their high replication and mutation rates. Due to this challenge to their

immune system, multicellular organisms have developed various distinct immune-recognition mechanisms. In animals classified as vertebrates, these mechanisms or systems can be simplified and classified as ‘innate’ and ‘adaptive’. Adaptive immune recognition is performed by a diverse array of antigen receptors, such as the T- and B-cell receptors (TCR and BCR), followed by the clonal selection and expansion of receptors with relevant specificities. This system is attributable for generating a memory in organisms, called the immunological memory, which enables a significant adaptive layout to exist in vertebrate animals (Akira and Takeda 2004). The adaptive immune response has two significant restrictions. First, the random creation of antigen receptors is unable to determine the origin, source, and biological context of the antigen for which they are specific. Second, a clonal distribution of antigen receptors requires specific clones to expand and differentiate into effector cells, before they can participate in host defense (Akira and Takeda 2004). This is the reason why the primary adaptive immune response is delayed, usually for 4–7 days, which is a huge delay with regard to the elimination of rapidly replicating microbes. However, the adaptive immune system does not function alone. The adaptive immune response is mediated by a combination of physiological signals that are provided by the innate immune system. The innate immune system identifies the presence and the nature of an infection, provides the first line of host defense, and mediates the determination and generation of molecules belonging to the effector class of the adaptive immune response. The innate immune system is fascinating with regard to its intricate pathways; new reports have continued to reveal new information about the innate immune system. Studies and new discoveries regarding

antimicrobial peptides, dendritic cells (DCs), and the complement system have all significantly contributed to the current knowledge regarding the innate immune system.

Efficient immune responses depend upon how close a connection and an orchestrate interaction are between the innate and adaptive immune system. γδ T cells and toll like receptors (TLR) serve as an important link between the innate and adaptive immune responses. γδ T cells play important roles in host defense against microbial infections, tumorigenesis, immunoregulation and development of autoimmunity. γδ T cells also have several innate cell-like characters that allow their early and rapid activation following recognition of cellular stress and infection. In order to accomplish these functions, γδ T cells use both the T cell receptor (TCR) and additional activating receptors (notably NKG2D, NOTCH, and TLR) to respond to stress-induced ligands and infection. γδ T cells express TLRs and modulate early immune responses against different pathogens (Akira and Takeda 2004).

The recent extraordinary discovery of pattern recognition receptors, including the TLRs, has renewed interest in the field of innate immunity. It is already known that these receptors have an important role in the recognition of a pathogen and stimulation of antimicrobial gene expression, and were able to mediate the functioning of the adaptive immune system. Current studies have shown that TLRs were also able to recognize certain ‘molecular signatures’ of microbial infection, trigger distinct signaling pathways, and mediate DC maturation and T helper cell differentiation (Akira and Takeda 2004).

Recognition by TLRs is based on the identification and recognition of byproducts released after microbial metabolism. Several metabolic pathways and gene products are unique to certain microbes and pathogens that are not encountered in host cells. Some of these pathways are responsible for housekeeping functions; their products are stored in a certain class of microorganisms and are crucial for their survival. For example, lipopolysaccharides (LPS), lipoproteins, peptidoglycans, and lipoteichoic acids (LTAs) are products released by bacteria, but are not produced by eukaryotic cells. These products are identified as molecular signatures of pathogens, and their recognition by the innate immune system produces signals regarding the presence of an infection. One peculiar aspect of innate immune recognition is that its targets are not completely identical between different species of microorganisms. There are various strain- and species-specific variations in the fine chemical structure; however, these are encountered in a common molecular pattern, which is conserved and invariant among microbes of a given class. For instance, the lipid-A sequence of LPS represents a consistent pattern observed in all gram-negative bacteria and triggers the pro-inflammatory response of LPS; counter intuitively, the O-antigen sequence of LPS is variable in different species of bacteria and cannot be identified by the innate immune system (Akira and Takeda 2004, Rakoff-Nahoum and Medzhitov 2009). Because the innate immune system can only recognize consistent and conserved molecular patterns, they are classified and named as PAMPs. The receptors of the innate immune system that are responsible for the recognition of PAMPs are named as PRRs. PAMPs have three similar aspects that make them ideal targets for identification by an innate immune system (Barton and Medzhitov 2002). First,

PAMPs are released only by microbes, and are not produced by host cells. Consequently, the recognition of PAMPs by the innate immune system allows a partial and initial distinction between ‘self’ and ‘microbial non-self’. Second, PAMPs are consistent and invariant among microbes of a given class. This allows a certain number of germ-line-encoded PRRs to perceive the presence of microbial infections. For example, the recognition of the conserved and consistent lipid-A pattern in LPS permits a single TLR to detect almost any gram-negative bacterial invader (Barton and Medzhitov 2002). Third, microbes cannot survive without their PAMPs. The loss of PAMPs or a mutation in their genes is either lethal for that class of microorganisms, or significantly reduces their number. Consequently, ‘escape mutants’ are not created. These unique characteristics of PAMPs show that they were recognized since the early period, during the evolution of host-defense immunological systems. In addition, several PAMPs are identified by the innate immune systems of mammals, invertebrates, and plants. It is necessary to highlight that PAMPs are not uniquely produced by pathogenic organisms; they can be produced by both pathogenic and non-pathogenic microorganisms (Barton and Medzhitov 2002). Notably, PRRs are not able to distinguish between pathogenic and commensal microorganisms. However, is vitally important to be able to distinguish between pathogenic and commensal microbes (Uematsu and Akira 2007). Humans are constantly exposed to commensal microflora, and the frequent activation and stimulation of inflammatory responses by commensals could potentially have lethal consequences for the host organism. However, this condition is not observed under homeostatic physiological conditions. The sophisticated mechanism that enables the host to tolerate

on-pathogenic microbes is largely unknown. Several hypotheses regarding the functioning of these hosts involve compartmentalization (confinement of microflora to the luminal side of the intestinal epithelium) and the use of anti-inflammatory cytokines (transforming growth factor-β (TGF-β) and interleukin (IL)-10 have an important role in the process) (Akira and Takeda 2004). The innate immune system uses several PRRs that are expressed on the cell surface, in intracellular compartments, or secreted into the blood stream and tissue fluids. The most important functions of PRRs include opsonization, complement and coagulation cascade activation, phagocytosis, pro-inflammatory signaling pathway activation, and apoptosis induction (Akira and Takeda 2004).

TLRs are PRRs that have a peculiar and very important function in animal immunity. TLRs belong to the type I transmembrane receptor family, which is composed of an extracellular leucine-rich repeat (LRR) domain and intracellular toll/IL-1 receptor (TIR) domain. LRRs are found in several sets of proteins, and recognize the ligands and agonists responsible for signal transduction. One of the characteristics of the LRRs is its sequence motif L(X2)LXL(X2)NXL(X2)L(X7)L(X2); in this consensus sequence, the “X” is an amino acid. LRRs and TLRs are separated from the transmembrane sequences by a ‘LRR carboxy-terminal domain’, which has the consensus motif CXC(X23)C(X17)C (Barton and Medzhitov 2002, Akira and Takeda 2004, Rakoff-Nahoum and Medzhitov 2009, Brown, Wang et al. 2011).

The TIR domain of toll proteins is a conserved protein–protein interaction module. In humans, the TIR domain is present in several cytoplasmic proteins, such as MyD88 (REFS 9–12) and TIRAP13 (TIR domain-containing adaptor protein); both are considered to be molecular adaptors that are involved in the signal transduction of TLRs (Barton and Medzhitov 2002).

The first discovered member of the toll family, Drosophila Toll, was discovered while observing maternal effects in fruitfly embryos. Other genes in this pathway encode the toll ligand Spätzle, the adaptor protein Tube, the protein kinase Pelle, the nuclear factor-**κ**B (NF-**κ**B)-family transcription factor Dorsal, and the Dorsal inhibitor and mammalian inhibitor of the **κ**B (I**κ**B) homologue Cactus (Barton and Medzhitov 2002, Kennedy 2010).

The similarities between the Drosophila Toll pathway and mammalian IL-1R pathway demonstrated that the toll pathway could potentially be involved in the immune system of the fruitfly, as well as in developmental patterning. This was observed in the genetically modified Drosophila, which rapidly developed a fungal infection, because of the failure to activate the production of the antifungal peptide Drosomycin. Additionally, fruitflies with loss-of function mutations in their Spätzle, tube, or pelle proteins were much more susceptible to fungal infections. Consequently, the toll pathway not only mediates dorsoventral pattern formation in embryos, but also mediates the antifungal immune defense in adult fruitflies (McNutt 1974, Akira and Takeda 2004).

Although the toll/NF-**κ**B pathways of Drosophila and mammals have several features in common, some differences also exist. One of the unresolved issues regarding the

immune system of the Drosophila involve the identities of pattern recognition molecules that activate the processing of Spätzle in response to fungal and gram-positive infections. Another is the identity of the receptor that mediates the activation of the Imd pathway in response to gram-negative bacterial infections. Because there are nine TLRs in Drosophila, it is possible that one of them was probably responsible for the activation of the Imd pathway (Akira and Takeda 2004).

There are at least ten TLRs in humans and mammalian species, and each of these have a distinct function with regard to the recognition of a ligand during an innate immune response. Many more ligands are yet to be identified, both for the TLRs that already have assigned ligands and those with no known ligands. TLR ligands are notably distinct and diverse with regard to their origin and structure. Most TLR ligands are consistent microbial products (PAMPs) that generate signals about the presence of an invader. All individual TLRs can probably recognize various structurally unrelated ligands. Some TLRs require additional proteins, in order to recognize their ligands. Despite the fact that the mechanism for ligand recognition is not completely known, studies have demonstrated that mammalian TLRs identify and recognize their ligands via direct binding, and therefore function as PRRs (Akira and Takeda 2004).

TLR4 was the first mammalian TLR to be found and characterized. It is expressed in several cell types, most commonly in macrophages and DCs. TLR4 recognizes LPS and activates signal transduction. The recognition of LPS by TLR4 requires several adaptor molecules. LPS binds to a serum protein, LBP (LPS-binding protein), which transfers LPS

monomers to CD14. CD14 is a high-affinity LPS receptor that can be found in the serum, or is expressed on the surface of macrophages as a glycophosphoinositol. CD14 is very important for the recognition of LPS, as CD14-deficient mice have a profound defect with regard to their responsiveness to LPS. MD-2, another accessory molecule of the LPS receptor, is a small protein that is expressed on the cell surface and is associated with the ectodomain of TLR4 (Akira and Takeda 2004).

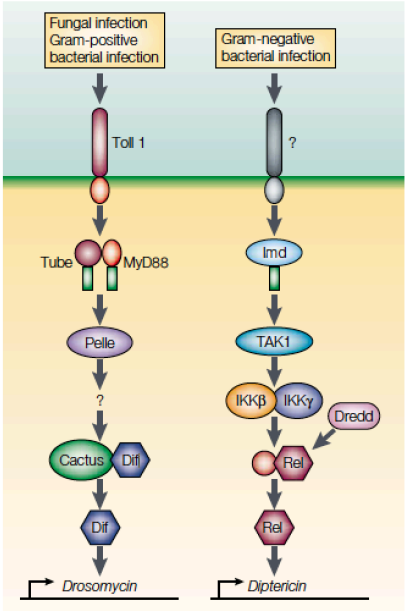

Figure 2: Toll like receptors and Imd pathways of drosophila:

The Toll pathway in the Drosophila is triggered by the presence of fungal and gram-positive bacteria. The activation of this pathway requires Tube and MyD88 adaptors, leading to degradation of Cactus, and the release of transcription factors of the nuclear factor-κB (NF-κB) family. The Imd pathway is activated in response to gram-negative bacterial infections. This pathway involves the Drosophila homologue of the protein kinase TAK1 (TGF-β-activated kinase), IKK-γ/IKK-β protein kinase complex, caspase Dredd, and NF-κB family transcription factor Relish (Akira and Takeda 2004).

RP105 is another accessory molecule that cooperates with TLR4 during LPS recognition; it is associated with MD-1, a homologue of MD-2.

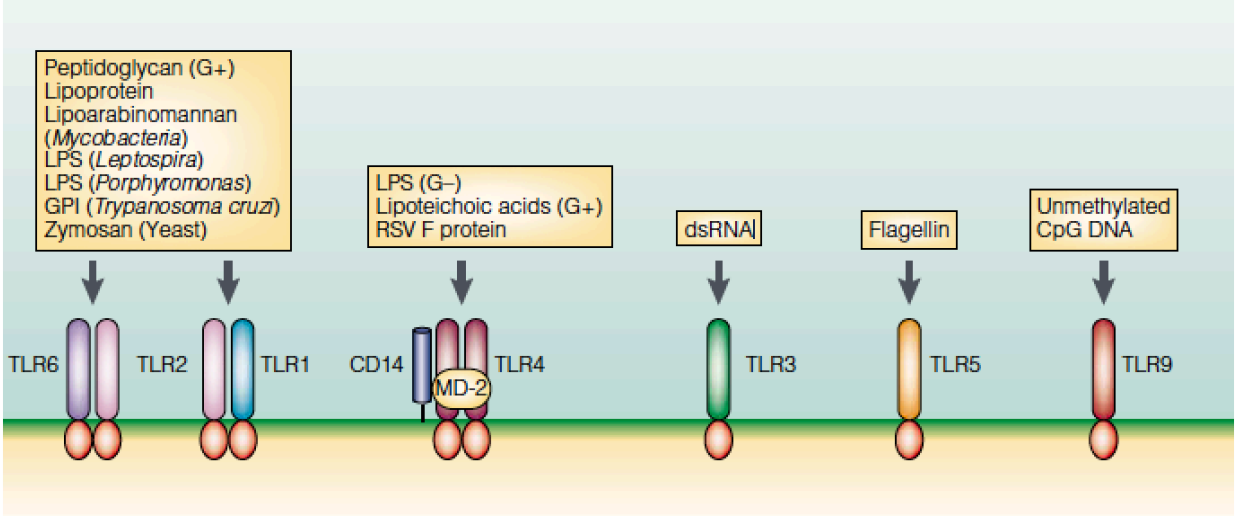

Figure 3: Toll-like receptors (TLRs) and their specific ligands:

TLRs recognize several pathogen-associated molecules. TLR4 recognizes lipopolysaccharides (LPS), while TLR2 recognizes a broad range of ligands after it forms dimers with TLR1 and TLR6. TLR3 is associated with the recognition of double-stranded RNA (dsRNA). TLR5 shows specificity towards bacterial flagellin. TLR9 acts as a receptor for unmethylated CpG motifs, which are abundant in bacterial DNA (Akira and Takeda 2004).

TLR4 also mediates the recognition of several other ligands such as LTA53, and a heat-sensitive cell-associated factor derived from *Mycobacterium tuberculosis*. TLR4 is also involved in the recognition HSP60, a heat shock protein (HSP). During the injury or lysis of cells infected with viruses, HSP60 can be released from necrotic cells. HSP60 might

have a role in enabling tissue remodeling and wound healing to occur after cell death (Akira and Takeda 2004, Teixeira, Zhao et al. 2019).

TLR5 mediates the recognition of flagellin, a conserved protein that forms bacterial flagella. Flagellin is a protein that does not undergo any process of posttranslational differentiation that could enable it to be distinguished from host cellular molecules.

The pattern of recognition of unmethylated CpG motifs in bacterial DNA by TLR9 is the most noteworthy. Unmethylated DNA i (the so-called 'CpG motif') has a potent immunostimulatory activity. Because cytosine methylation does not occur in bacteria, and most CpGs in the mammalian genome are methylated, CpG motifs might signal the presence of microbial infections (Akira, Uematsu et al. 2006).

Recognition of Pathogens by TLRs:

Nowadays, significant levels of effort and several studies have been directed towards understanding the complex signal transduction pathways that are activated by TLRs. The transcription factor NF-κB is an important regulator that induces the expression of key pro-inflammatory mediators and cytokines that contribute to an immune response. NF-κB is a hetero or homodimeric transcription factor that binds to the promoter of a wide range of different target genes. TLR signaling cascades lead to IkB phosphorylation, ubiquitination, and proteasomal degradation of this protein, which consequently enables the release of NF-κB dimers. The phosphorylation of IkB is performed by the IkB kinase complex, a large multi-subunit complex consisting of at least two catalytic subunits and a

regulatory subunit. The induction of NF-κB -dependent gene expression generates strong pro-inflammatory responses. Genes activated by NF-κB are upstream activators of NF-κB, and lead to the amplification of the host defense response to microbial invaders. Pro-inflammatory gene expression is mediated and controlled by TLRs, and is also regulated by the activation of mitogen-activated protein kinases. This leads to the phosphorylation of multiple proteins and several transcription factors, which consequently induces the release of pro inflammatory cytokines. TLRs acquire intracellular adaptor molecules and kinases, to enable them to transduce their signals and activate immune responses. For example, the TIR domain of the adaptor molecule MyD88 is associated with the TIR domain of all TLRs. The MyD88 adaptor is mostly necessary for transmitting signals to NF-κB /mitogen-activated protein kinase pathways. MyD88 recruits the IL-1 receptor-associated kinase, which then induces the activation of tumor necrosis factor receptor-associated factor 6, and the NF-κB and mitogen-activated protein kinases. Although MyD88 is considered to be a universally used adaptor protein for all TLRs, recent studies have revealed the existence of other adaptors, such as TIRAP, TRAM, and TRIF (Akira, Yamamoto et al. 2003, Akira, Uematsu et al. 2006, Xia, Winkelmann et al. 2013, Qin, Li et al. 2016).

Myd88-Dependent and Myd88-Independent Pathways:

During infections, multiple TLR pathways are activated by different ligands, which are components of microbes that activate or trigger the immune system. The interplay between TLR signaling pathways could have important effects on host inflammatory

responses. Gram-negative bacteria release peptidoglycan-associated lipoprotein (PAL), which is a TLR2 agonist, in conjunction with bacterial proteins and LPS into the blood of septic animals and human serum. PAL and LPS synergistically activate the generation of immune responses. It is important to understand the mechanism of response of TLRs, as the ligands, duration for which cells are challenged, and concentrations of these bacterial products would play a role in the immune response and the outcome (Akira and Takeda 2004, Bagchi, Herrup et al. 2007). MyD88 is an adaptor molecule used by most TLRs, which are generated via two intracellular pathways, i.e. the MyD88-dependent (D) and the MyD88-independent (I) pathways. When the ligand triggers a TLR, MyD88 engages with the internal portion of the TLR and downstream signaling is propagated. Studies have identified two intracellular TLR pathways. The D pathway requires the adaptor molecule MyD88 to propagate signals and induce the expression of pro-inflammatory cytokines such as TNF and IL8 (Bagchi, Herrup et al. 2007, Xia, Winkelmann et al. 2013). The I pathway generates signals through the Toll-IL-1R domain-containing adaptor-inducing IFN-β, which leads to IFN-β production. The I pathway also activates NF-**k**B; however, this activation process is delayed and also leads to the production of TNF and other inflammatory cytokines. All TLRs use the MyD88-dependent pathway, which requires an MyD88 adaptor molecule, with the exception of TLR3 and TLR4, which use the MyD88-independent pathway. In the dependent pathway (D), TLRs require the MyD88 adaptor molecule and in the independent pathway (I), TLRs do not require MyD88 as an adaptor molecule. To date, TLR4 is the only TLR that activates both the D and I pathways. It has been recently reported that TLR3, which recognizes viral dsRNA and induces

inflammatory cytokines, such as TNF and IL-6, can only use the independent pathway (I). TLR3 has been reported to induce IFN-β production via the I pathway; however, additional pathways that mediate the induction of IFN- β by dsRNA have also been identified (Bagchi, Herrup et al. 2007). To date, no clear explanation has been provided about how synergy, priming, and tolerance between TLR agonists occur (Bagchi, Herrup et al. 2007).

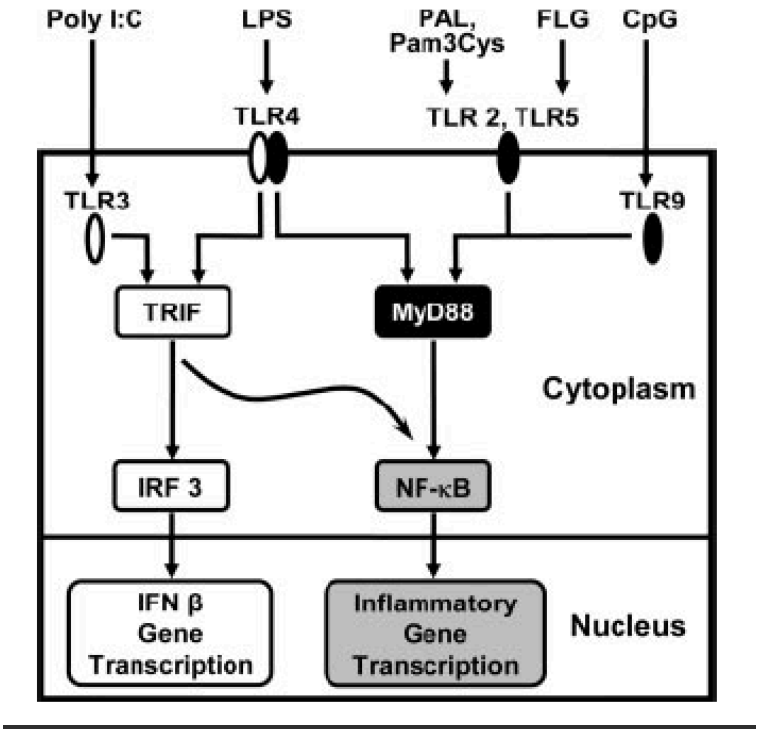

Figure 4: TLRs and TLR agonists corresponding to I and D pathways

The D pathway is demonstrated using a black background and white text, while the I pathway is shown using a white background and black text. TLR4 has affects both D and I pathways. The curved arrow indicates that both the D and I pathways induce inflammatory cytokines through NF-κB (Bagchi, Herrup et al. 2007).

TLR2 and TLR3:

TLR2 is responsible for the recognition of bacterial and fungal components such as peptidoglycans from gram-positive bacteria, bacterial lipoproteins, mycobacterial cell-wall lipoarabinomannans, glycosylphosphatidylinositol lipids from Trypanosoma Cruzi, a phenol-soluble modulin produced by *Staphylococcus epidermidis*, and yeast cell walls. TLR2 functions as a receptor and can identify and recognize LPS produced by *Leptospira interrogans* and *Porphyromonas gingivitis*, both of which are structurally very distinct from gram-negative LPS. This vast range of ligands recognized by TLR2 can partially explain the association of TLR2 with TLR1 and TLR6. Hence, the formation of heterodimers between TLR2 and either TLR1 or TLR6 determines the specificity of ligand recognition. For example, TLR2 cooperates with TLR6 for the recognition of the mycoplasmal macrophage-activating lipopeptide with a weight of 2 kDa. In this case, TLR6 is responsible for discriminating between bacterial lipoproteins, which are triacylated at the amino-terminal cysteine residue, and the diacylated mycoplasmal lipoprotein MALP-2. It is not completely clear if TLR2 heterodimerization is induced by appropriate ligands or if it occurs before the ligand interacts with the receptor (Bagchi, Herrup et al. 2007, Benakanakere, Li et al. 2009, Blednov, Black et al. 2017, Chen, Xie et al. 2017). One interesting aspect is that TLR1 and TLR6 are expressed on many cell types; however, the expression of TLR2 is restricted to antigen-presenting cells and endothelial cells. The combination of TLR2 with TLR1 or TLR6 provides an important mechanism for mediating the cellular responsiveness to microbial products. The complete repertoire of possible TLR heterodimers is not completely known; however, TLR4 and TLR5 seem to function

as homodimers (Burns, Bachrach et al. 2006, Sahoo, Basu et al. 2013, Kennedy, Najdovska et al. 2014, Mortazavi, Amin et al. 2015).

TLR3 has two interesting characteristics that make it unique among mammalian TLRs. TLR3 does not contain a conserved proline residue in the position equivalent to that of proline-712 of mouse TLR4, which results in the unresponsiveness of TLR3 to LPS. Interestingly, TLR3 is expressed predominantly in dendritic cells. Current studies show that TLR3 acts as a cell-surface receptor for dsRNA. dsRNA is a molecular pattern produced by most viruses during their infection cycle. The synthetic analogue of dsRNA, known as polyinosine-polycytosine (poly I:C), activates TLR3 when cells are stimulated with it. Cells with a TLR3 deficiency do not respond to Poly I:C, as well as to viral dsRNA. The contribution of TLR3 to immune defense against viruses is not fully understood; however, TLR3 is strongly activated when stimulated with viral dsRNA in epithelial cells, which represent the first line of defense. In our studies, when epithelial cells are challenged with Poly I:C, TLR2 is sometimes upregulated significantly more than TLR3 (Bakaysa, Potter et al. 2014). HMGB1 and Hsp60 seem to activate TLR2 through TLR3 stimulation. This observation suggests the occurrence of crosstalk between TLR2 and TLR3, and that HMGB1 and Hsp60 play a role in activating TLR2 that has never been demonstrated before (Chalmers, Eidelman et al. 2013, Singh, Biswas et al. 2016, Al-Ofi and Al-Ghamdi 2018, Martinus and Goldsbury 2018).

Role of HMGB1 and Hsp60 (endogenous ligands) in TLR activation:

Previous studies have shown that damaged and necrotic cells passively release HMGB1, which activates TLR2. In injury models, Hsp60 and HMGB1 have been shown to increase TLR2 signaling. The protein encoded by HSP60 is a member of the chaperonin protein family. This protein is implicated in mitochondrial protein import and microcellular assembly. HSP60 facilitates the correct folding of proteins imported from the cytoplasm and assists in their transportation as well. HSP60 also prevents misfolding and promotes the refolding or assembly of unfolded and stress-denatured mitochondrial proteins. The increase in protein expression is regulated transcriptionally, and the upregulation of HSP60 is a crucial part of the stress response of cells. The role of HSP60 in performing cellular activities and maintaining integrity is crucial. Knockouts lead to multitudes of negative cellular complications and the effect of HSP60 on these cellular processes is not always fully understood. HSP60 knockout in adult mouse heart resulted in altered mitochondrial complex activity, mitochondrial membrane potential, ROS production, and eventually led to dilated cardiomyopathy, heart failure, and lethality (Chalmers, Eidelman et al. 2013, Singh, Biswas et al. 2016, Al-Ofi and Al-Ghamdi 2018, Hu, Chen et al. 2018, Martinus and Goldsbury 2018).

In the cytosol, HMGB1 promotes autophagy and the recruitment of the myddosome complex to Toll-like vesicular receptor compartments. Outside the cell, it binds to specific receptors, or with high affinity to DNA, nucleosomes, IL-1β, lipopolysaccharides, and lipoteichoic acid, to mediate responses under certain physiological or pathological

conditions. Known receptors for HMGB1 include TLR2, TLR4, the receptor for advanced glycation end products, CD24-Siglec G/10, chemokine CXC receptor 4, and TIM-3 (Chalmers, Eidelman et al. 2013).

The bioactivity of extracellular HMGB1 is determined by the extent of modification of conserved redox sensitive cysteine residues (C23, C45, and C106). The molecular conformation of these cysteine residues allows HMGB1 to bind and signal to cells via the TLR4/MD-2 complex, to induce cytokine release in macrophages. The role of HMGB1 in inflammation and immunity is determined by its post translational modifications. The post-translational acetylation of lysine residues within nuclear localization signals of HMGB1 promotes the inflammation and hyperacetylation of HMGB1, shifts its equilibrium from a predominant nuclear location toward a cytosolic and subsequent extracellular presence failure, and enhances its lethality. We hypothesize that the stimulation of epithelial cells with Poly I:C would trigger the TLR3-HMGB1-Hsp60-TLR2 pathway and reveal the crosstalk of signals between TLR2 and TLR3.

Summary:

The recognition of a pathogen is essential for initiating an innate immune response. This recognition occurs via germline-encoded pattern-recognition receptors. We have chosen to focus upon TLRs, which are pattern recognition receptors. They are responsible for recognizing PAMPs.

The current studies separate TLR2 and TLR3 signaling pathways. During the development of drugs, we should consider the response of these TLRs together.

After identifying TLR3 as one of the important receptors in the inflammatory cytokine network of HGECs, we hypothesized that TLR3 downstream signaling might control the activation of other TLRs via the MyD88-dependent expression of endogenous TLR ligands.

Specific Aims:

Aim 1:

To determine the association between TLR2 and TLR3 in vitro

To determine the role of the Myd88-TLR3 pathway in vitro

To determine the role of HMGB1 and Hsp60 in the TLR2 pathway signalizing in vitro

Aim 2:

To test the hypothesis that the TLR3-dependent augmentation of pro-inflammatory cytokine production involves MyD88 *in vivo*

Chapter 2: Papers and Manuscripts accomplished during the DScD program:

IFN-β secretion is through TLR3 but not TLR4 in human gingival epithelial cell

Hellen Teixeira[1], Jiawei Zhao[2,3], Denis F. Kinane[4], Manjunatha R. Benakanakere[2,*]

[1]Department of Orthodontics, School of Dental Medicine, University of Pennsylvania, Philadelphia PA 19004

[2]Department of Periodontics, School of Dental Medicine, University of Pennsylvania, Philadelphia PA 19004

[3]Present address: Department of Pathology, Wayne State University School of Medicine, 541 East Canfield Ave., Scott Hall 9215, Detroit, MI 48201

[4]Division of Periodontology, School of Dental Medicine, University of Geneva Faculty of Medicine, Geneva, Switzerland.

Key words: Toll-like receptors, Type I Interferon, Human Gingival Epithelial Cells, Interferon regulatory factor.

This chapter was published in *Molecular Immunology* Journal- 2019

The role of MyD88 in MyD88-independent TLR3 signaling pathway

Hellen S. Teixeira[1], Jiawei Zhao[2,3] and Manjunatha R. Benakanakere[2*]

[1]Department of Orthodontics, School of Dental Medicine, University of Pennsylvania, Philadelphia PA 19004

[2]Department of Periodontics, School of Dental Medicine, University of Pennsylvania, Philadelphia PA 19004

[3]Present address: Department of Pathology, Wayne State University School of Medicine, 541 East Canfield Ave., Scott Hall 9215, Detroit, MI 48201

Keywords: Human gingival epithelial cells, TLR3, MyD88, HMGB1, Hsp60, Pro-inflammatory cytokine

This manuscript will be submitted to the journal *Cells*

Chapter 3: Materials and Methods

Cell challenge assays:

Primary human gingival epithelial cells (HGECs) were isolated from the same cell donor and experiments repeated 3 times after obtaining approval from the Institutional Review Board, at the University of Pennsylvania. HGECs were harvested at the 3rd passage step, and seeded at a density of 0.5 × 10^5 cells/well in 6-well culture plates, according our previously published method. The cells were maintained in 2 ml of complete medium until they attained 80% confluency and then washed twice with fresh medium and maintained in 2 ml of plain medium. At 90% confluency, the cells were incubated with a panel of bacterial and viral ligands (live *Porphyromonas gingivalis,* heat killed *P. gingivalis (P.g),* Pam3CSK4, *P.g* LPS; Poly I:C, *E. coli* K12 LPS, Flagellin from *Salmonella typhimurium,* FSL1, Imiquimod, and ODN) for 24 hours, as described previously (Livak and Schmittgen 2001). After stimulating cells for 24 hours, the supernatant was collected and TNF ELISA was performed. In later experiments, cells were either stimulated with 1 µg/ml FSL-1 (TLR2/6 ligand), 5 µg/ml of Poly I:C (TLR3 ligand), or 1 µg/ml Pam3CSK4 (TLR1/2 ligand) (Invivogen, CA), based on the results of an initial dose response and agonist screening process. Culture supernatants were collected at the end of the experiment and stored at -80 °C until further use. The production levels of IL-8 and TNF were determined by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (R&D systems). None of the agonist stimuli affected cell viability, as shown by the results of the trypan blue exclusion process, performed as reported previously (Teixeira, Zhao et al. 2019).

RNAi:

SMARTpool siTLR2, siTLR3, and MyD88 ON-TARGETplus SMARTpool and ON-TARGETplus non-targeting siRNA were obtained from Dharmacon. HGECs were transfected using the siPort NeoFx transfection reagent, according to the manufacturer's instructions (Ambion). Briefly, 100 nM of siRNA was used to transfect cells that had achieved 60%–70% confluency. Cells were stimulated with Poly I:C 24 h-post transfection, and harvested after another 24 h (Livak and Schmittgen 2001).

Real-time PCR:

cDNA was prepared using the cDNA archive kit (Thermo Fisher), from total RNA sequences extracted from cultured cells using the RNeasy mini kit (Qiagen). Real-time PCR was performed using the TaqMan technique with 50 ng of cDNA on the 7500 Fast system (Applied Biosystems). TLR1, TLR2, TLR3, TLR6, IL-8, TNF, HMGB1, Hsp60, and GAPDH primers and probes were prepared as described previously. The quantitative TaqMan PCR-Array was custom designed, based on our previously published microarray data regarding HGECs. HGECs were grown till they achieved confluency, and stimulated with Pam3CSK4 (1 μg/ml), FSL-1 (1 μg/ml), or Poly I:C (5 μg/ml) for 30, 60, 90, 120, and 240 minutes, and 4 and 24 hours. The fold increase was determined by the ΔΔCT method (Livak and Schmittgen 2001). The mean log fold increase was used to derive a heat map with two-way hierarchical clustering (rows=genes, columns=samples), using the MeV v4.1 software, as shown previously (Livak and Schmittgen 2001).

TNF and IL-8 analysis by ELISA:

TNF and IL-8 levels in the supernatants of HGECs were determined by using the Quantikine ELISA kit (R&D Systems Inc.). All experiments were performed in triplicate (Bagchi, Herrup et al. 2007, Teixeira, Zhao et al. 2019).

Animal Model:

The animal study was conducted to test the hypothesis that the TLR3-dependent augmentation of pro-inflammatory cytokine production involves MyD88 in vivo. Experiments were performed using 8 adult female C57BL/6J mice (14–16 g) per group, with a protocol approved by the University of Pennsylvania Institutional Animal Care and Use Committee. The rostral back of the animals was trimmed with an electric shaver and subsequently removed. Poly I:C was topically applied on mice for 6, and 12 hours. A chromophore containing 25 and 8 mg of Poly I:C was applied on the shaved skin of the back (2.5 cm × 2 cm); the best time point and concentration were selected (8mg for 12 hours) based on the preliminary results. Control mice were treated similarly with a control vehicle Chromophore (Alkanani, Hara et al. 2014, Sakai, Sanders et al. 2016, Blednov, Black et al. 2017, Hu, Chen et al. 2018).

Animals were euthanized under isoflurane sedation, and their skin was acutely dissected (Blednov, Black et al. 2017). Skin was fixed in 4% paraformaldehyde, followed by 30% sucrose, frozen in an optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek, Torrance, CA), cut in 8-μm sections on a cryostat, and stored at -80 ºC. The

slides were defrosted the next day and fixed in 10% formaldehyde for 10 minutes. After an appropriate period, the slides were immersed in methanol at -20 °C for 5 minutes, and then washed in PBS three times, for 5 min each. Samples were blocked in 10% horse serum for 30 minutes at room temperature, and again washed in 1x PBS. Primary antibodies (p-IRF-3 (S536), P-NF-KappaB p65 (S536), Isotype and TNF alpha from CellSignaling®) were added and the reaction mixture was incubated in a humid chamber overnight at 4 ºC (primary antibodies were diluted 1:800 and 1:100 times, according to the manufacturer's instructions, using 0.5% horse serum (Xia, Winkelmann et al. 2013, Hu, Chen et al. 2018). On the next day, samples were incubated with a secondary antibody (Alexa Fluor from CellSignaling®) in a humid chamber for 1 hour at room temperature. Secondary antibodies were diluted 1:100 or 1:200 (concentration used) times using 0.5% horse serum). The slides were mounted with a cover slip using Prolong® Gold anti-fade reagent with DAPI and mounting media (P-36931, Life Technologies™), and stored for 20 minutes in a dark drawer. The edges were sealed with nail vernix and slides were observed under a microscope (Sakai, Sanders et al. 2016).

All images for each specific anti-body were capturing using the same capture time.TNF and GRO capture time was 10s using the Z-stacking technology. NFkb and IRF capture time images were 15 s with Z-stacking technology.

Images captured from 3–4 skin sections obtained from each animal were imaged at a magnification of 60X. Images were evaluated by a trained observer blinded to the treatment conditions. The intensity of immunofluorescence within the epidermis was

measured using Image J (subtracting background fluorescence), and the mean immunofluorescence was determined for each mouse (8 mice per group) (Gonzalez-Barranco, Sandoval-Islas et al. 1978, Marcelli Barge, Benajam et al. 1979, Szaleczky, Pronai et al. 2000, Niflioglu and Lebe 2014).

Statistical Analysis

Statistical analysis was performed using Prism 6.0 (GraphPad). Data were analyzed via one-way ANOVA, followed by Tukey's multiple comparison tests.

Preliminary Data (Pilot Study):

A pilot study was performed using 20 mice (5 per group). The rostral back of the animals was trimmed with an electric shaver and subsequently removed. Poly I:C was topically applied on mice for 6, and 12 hours. A chromophore containing 25 and 8 mg of Poly I:C was applied on the shaved skin of the back (2.5 cm × 2 cm); the best time point and concentration were selected (8mg for 12 hours) based on the preliminary results.

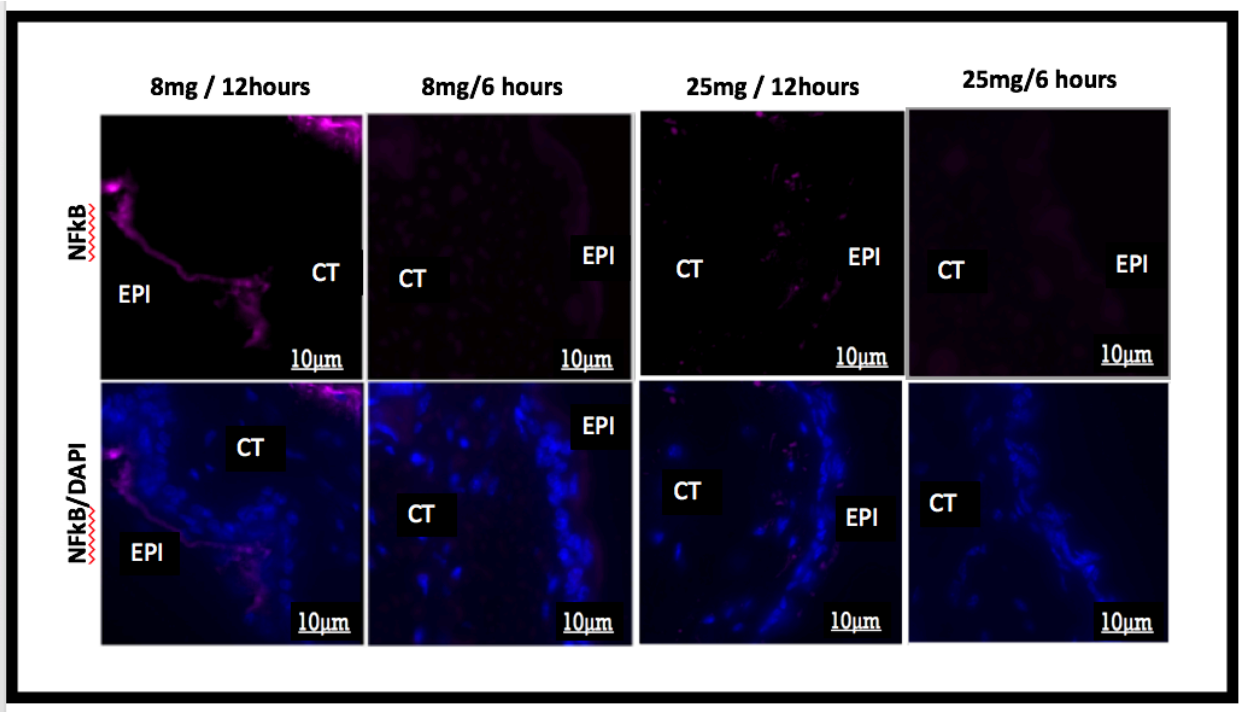

Figure 5: Epithelial cell staining for the visualization of phosphorylated NF-κB transcriptional factors in wild type mice versus MyD88 knockout mice.

Epithelial cells from wild type mice were challenged with 8mg and 25mg of Poly I:C at 6 and 12 hour's timee points. Immunofluorescence was observed because of the P-NF-kappaB p65 (S536) in skin tissue sections treated with Poly I:C. A higher mean of fluorescence intensity was observed on the surface skin of the mouse treated with 8mg of Poly I:C for 12 hours (pink [P-NF-kappaB p65]; blue [DAPI]).(Original magnification 60x). EPI, epithelium;CT, connective tissue. (N = 5 mice per group. Bar = 10 µm).

Control mice were treated similarly with a control vehicle Chromophore (Alkanani, Hara et al. 2014, Sakai, Sanders et al. 2016, Blednov, Black et al. 2017, Hu, Chen et al. 2018). High expression of Phospho-NFkB colocalization was encountered on animal treated with 8mg of Poly I:C for 12 hours.

Chapter 4: Results

We have previously shown that the activation of TLR3 in HGECs induces robust pro-inflammatory responses that are mediated by the mTOR signaling network (Teixeira, Zhao et al. 2019). Here, we further investigated whether other TLR signaling pathways could contribute to this robustness in the activation of the pro-inflammatory network in HGECs. Similar to the previously examined time-dependence of treatment, the early and late activation of the inflammatory response was investigated 30, 60, 90, and 120 minutes, as well as 4 and 24 hours after stimulation with ligands. We observed that Poly I:C induced a higher expression of TLR2, TLR4, and TLR7, as compared to that induced by FSL-1 and LPS at earlier time points. In comparison to other ligands, Poly I:C also induced the robust activation of pro-inflammatory genes and their expression **(Figure 6)**.

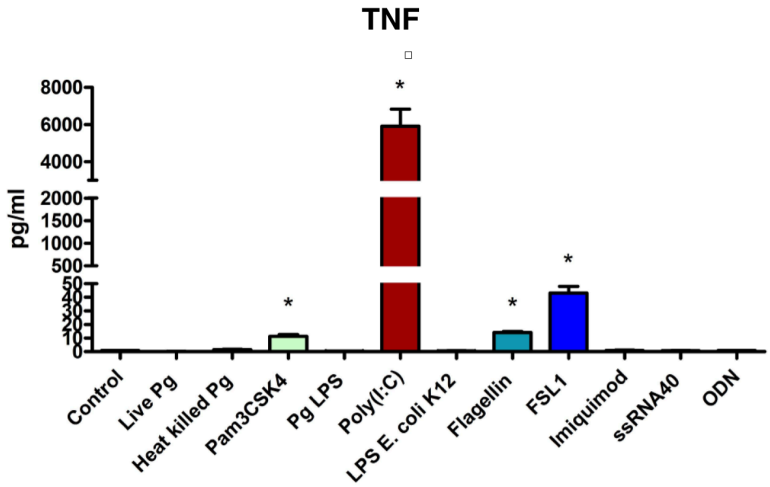

Figure 6: TLR3 stimulation of HGECs leads to robust activation of TLR signaling:

Cells were treated with various TLR ligands for 24 h and the supernatant was subjected to TNF measurement using ELISA. TLR3 stimulation occurred via the Poly I:C induced robust generation of TNF

cytokines. (Data represented as mean ±SE of three independent experiments using primary epithelial cells from a donor).

Surprisingly, cells stimulated with Poly I:C for 24 hours induced significantly higher levels of TLR2 **(Figure 6).** Further, to investigate the cytokine production after 24 hours, HGECs were treated with a panel of TLR ligands and examined for TNF production. Poly I:C induced higher levels of TNF production, as compared to that of other TLR ligands (**Figure 7**), which is in agreement with our previous observation. TLR2 can heterodimerize with TLR1 or TLR6 to induce Myd88-mediated signaling. To check if there is any difference in the expression levels of TLR1, TLR2, and TLR6, HGECs were treated with Pam3CSK4 (TLR1/2), FSL-1 (TLR2/6), and Poly I:C (TLR3) ligands for 24 hours and examined using quantitative real-time PCR.

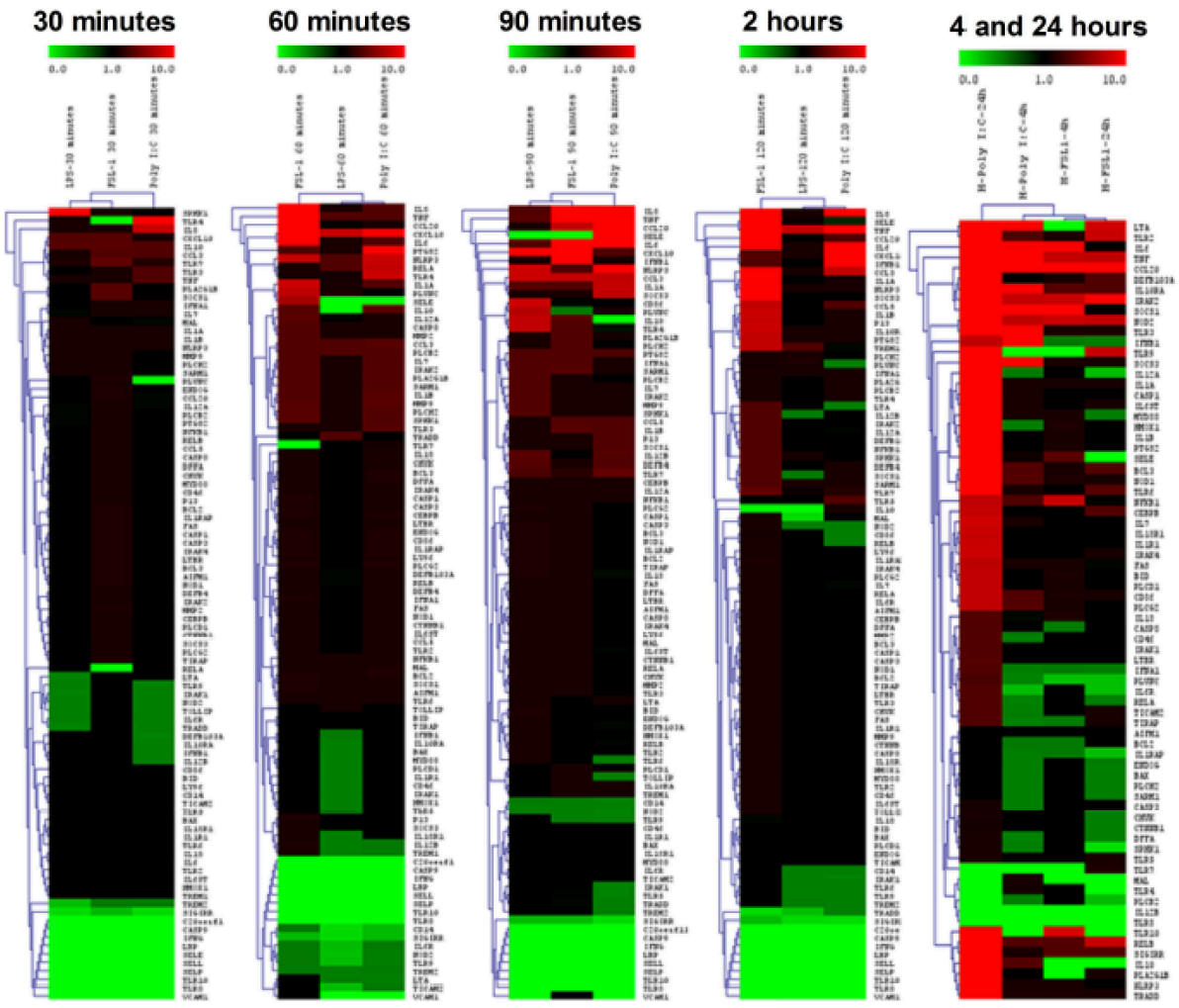

Figure 7 : Stimulation of HGECs with TLR3 leads to robust activation of TLR2 TNF and IL-8 signaling:

HGECs were incubated with *E. Coli* LPS (1 µg/ml), FSL-1 (1 µg/ml) and Poly I:C (5 µg/ml) for 30, 60, 90, 120 minutes, and 4 and 24 hours. We isolated total RNA, converted it to cDNA (pooled from three independent experiments), and analyzed customized qPCR-arrays. The ΔΔCT values were used to generate the heatmaps based on two-way hierarchical clustering, using MeV v4.1 software (*rows*=genes, *columns*=samples). The *color scale* indicates certain relative expression levels: *Red*, above mean; *green*, below mean; and *black*, below background. Statistical comparison was performed after ELISA via one-way ANOVA, followed by Tukey's multiple comparison test (*$p < 0.05$). Results are represented as mean ± SE values.

Unexpectedly, Poly I:C induced higher TLR2 expression levels than TLR3 (**Figure 8**). Because the level of TLR2 gene expression and TNF production after providing Poly I:C stimulation was significantly higher at 24 hours, we hypothesized that the production of robust pro-inflammatory mediators via TLR3 signaling occurs partly through the activation of TLR2.

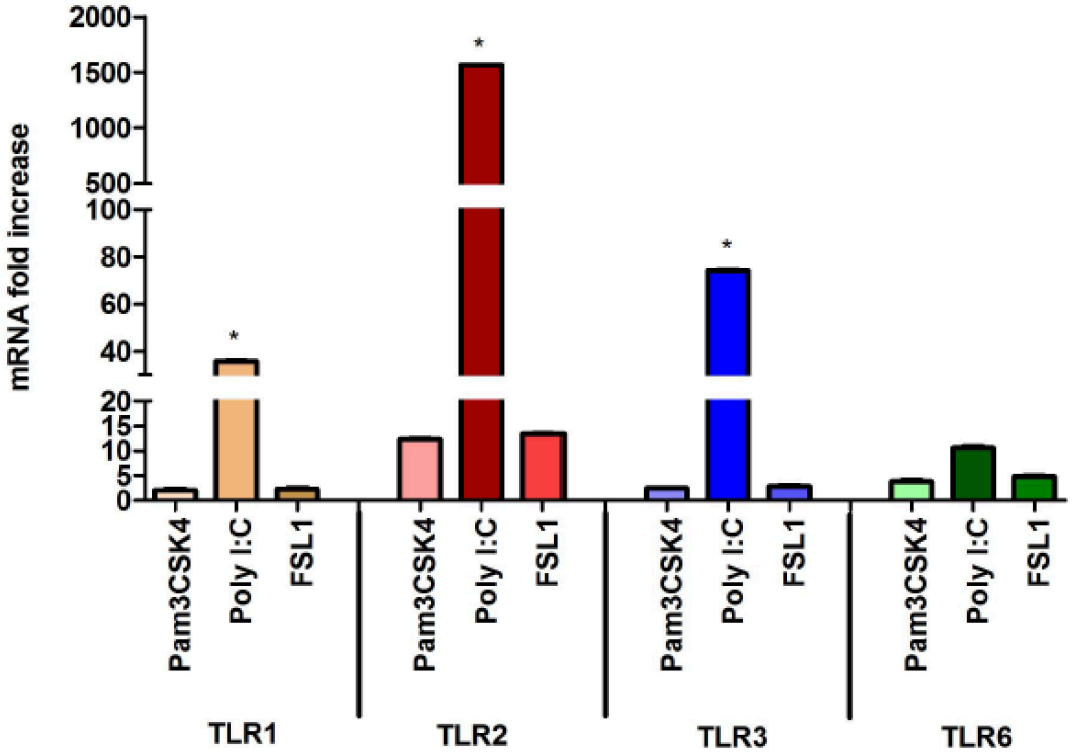

Figure 8: TLR3 stimulation induced high levels of TLR2 gene expression:

HGECs were treated Pam3CSK4 (1 µg/ml), FSL-1 (1 µg/ml), and Poly I:C (5 µg/ml) for 4 and 24 hours. Quantitative real-time PCR was performed for cDNA, as stated above. Poly I:C induced higher levels of TLR3 gene expression at 4 hours, but at 24 hours post stimulation, TLR3 activation induced significantly

higher levels of TLR2 gene expression. Statistical test: We performed one-way ANOVA, followed by Tukey's multiple comparison test (*p < 0.05). Data represented as mean ±SE of three independent experiments using primary epithelial cells from a donor.

TLR3-mediated expression of TLR2 requires MyD88:

To test whether TLR3 can impact the expression levels of TLR2, TLR3 was down-regulated by siRNA and HGECs were stimulated with Poly I:C. As shown in **Figure 9**, the silencing of *TLR3* led to a significant down-regulation of TLR2. Conversely, the silencing of *TLR2* and subsequent stimulation with FSL1 had no effect on the TLR3 expression levels (**Figure 9**).

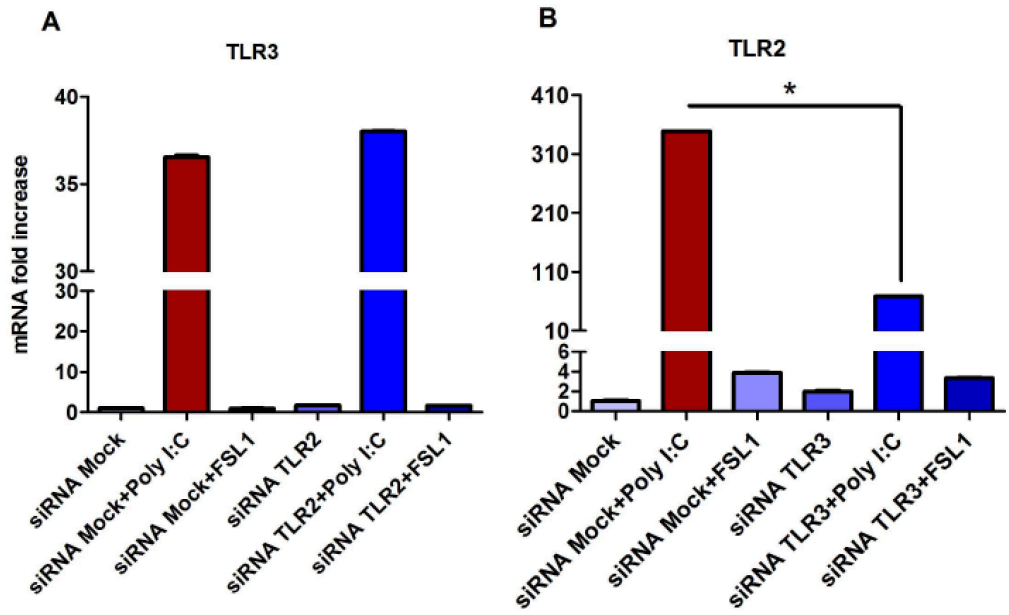

Figure 9 :Silencing of TLR3 reduced TLR2 gene expression in HGECs:

Epithelial cells were challenged with Poly I:C and FSL1. When *TLR3* was silenced, the expression of TLR2 was significantly downregulated after stimulation with Poly I:C (A). On the other hand, the silencing of *TLR2* had no effect on the expression of TLR3 (B). Statistical test: We performed one-way ANOVA, followed by

Tukey's multiple comparison test (*p < 0.05). Data represented as mean ±SE of three independent experiments using primary epithelial cells from a donor.

It is well-documented that TLR2 signaling requires MyD88 adapter molecules, and that TLR3 does not require MyD88 to induce downstream signaling (Bagchi, Herrup et al. 2007, Xia, Winkelmann et al. 2013, George, Kim et al. 2017). Therefore, we investigated whether the ability of TLR3 to impact TLR2 expression is affected by the presence of MyD88. To accomplish this, MyD88 was silenced by siRNA and HGECs were subsequently stimulated with Poly I:C for 24 h. Accordingly, the silencing of *MyD88* down-regulated TLR2 expression levels, while no changes were observed in TLR3 levels (**Figure 10A and 10B**). Together, our data demonstrates the necessity of MyD88 for the TLR3-regulated expression of TLR2.

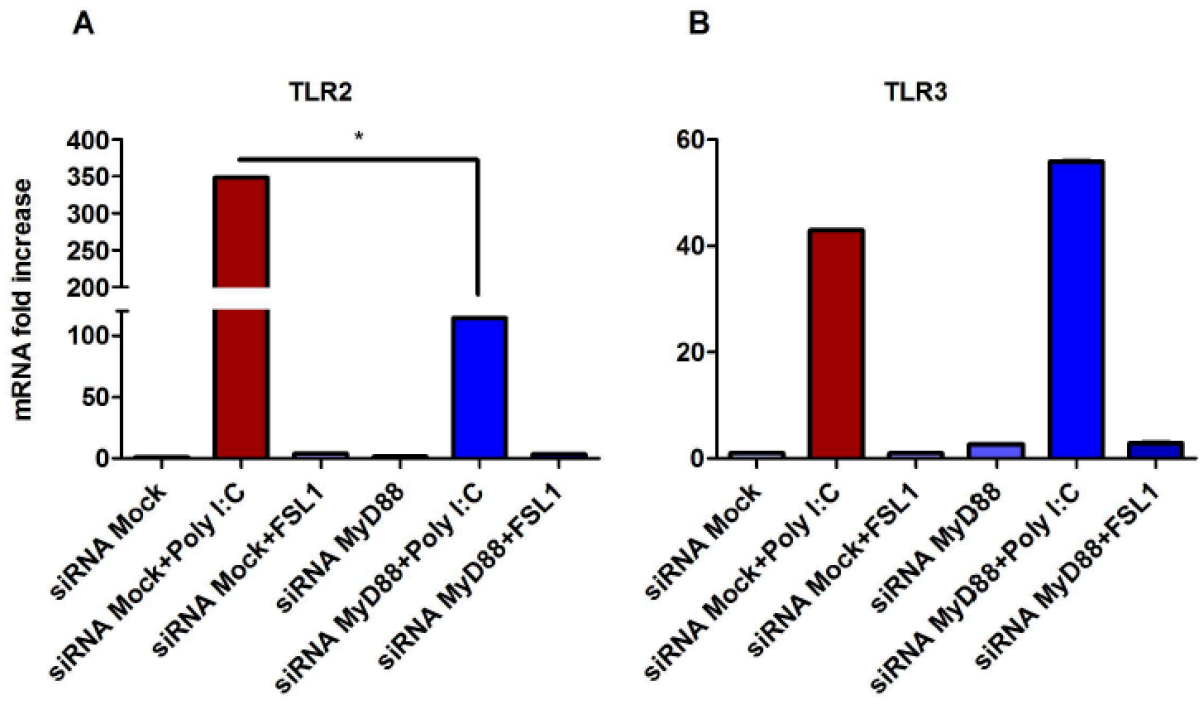

Figure 10: Up-regulation of TLR2 by Poly I:C is partly mediated by MyD88:

Epithelial cells were challenged with Poly I:C and FSL1. When *MyD88* is silenced, the expression of TLR2 significantly decreased after stimulation with Poly I:C. (A). However, the silencing of *Myd88* did not alter the expression of TLR3 (B). Statistical test: We performed one-way ANOVA, followed by Tukey's multiple comparison test (*p < 0.05). Data represented as mean ±SE of three independent experiments using primary epithelial cells from a donor.

TLR3 signaling increases pro-inflammatory cytokine secretion partially through the activation of TLR2 and MyD88.

To provide insight into the role of MyD88, we investigated the differential expression profiles of TLR2, TLR3, and MyD88, after silencing genes using siRNAs and subsequently providing exposure to their respective ligands. First, we examined the silencing of *TLR2*. As expected, TLR2 expression levels were minimal in the absence and

presence of its ligand, FSL-1 (**Figure 11A**). Interestingly, stimulation with the TLR3 ligand, Poly I:C, was capable of restoring the expression levels of TLR2 more robustly than that with FSL-1 alone **(Figure 11A).** Additionally, the silencing of *TLR3* and subsequent exposure to Poly I:C resulted in a 2-fold increase in the expression of MyD88. **(Figure 11C).**

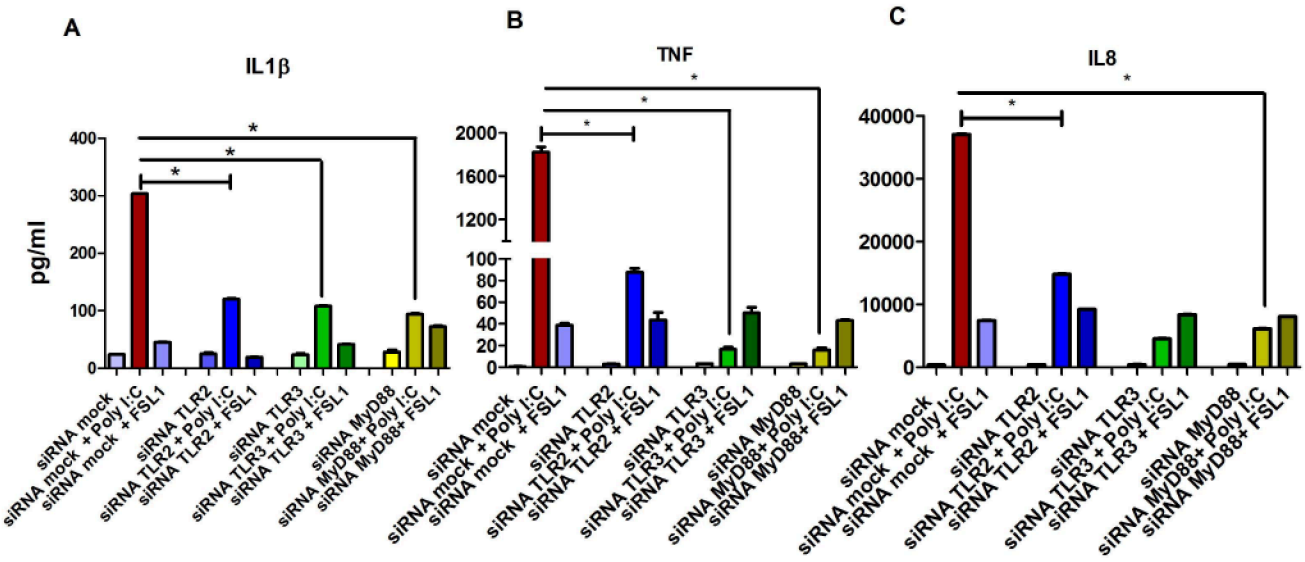

Figure 11: TLR3 stimulation induces MyD88 production and its inhibition attenuates its expression:

HGECs were challenged with Poly I:C and FSL1. were treated with respective ligands after silencing *TLR2*, *TLR3*, and *MyD88*. The silencing of *TLR3* induced a significantly higher expression of TLR2, even when *TLR2* was silenced (A). As expected, siTLR3-treated cells down-regulated TLR3 (B). Interestingly, Poly I:C treated cells significantly increased Myd88 expression levels (C). However, when *MyD88* is silenced, Poly I:C significantly down-regulated MyD88 expression. The extent of TLR2 upregulation by Poly I:C was higher than that of TLR3 upregulation by FSL-1. Statistical test: We performed one-way ANOVA, followed by Tukey's multiple comparison test (*p < 0.05). Data represented as mean ±SE of three independent experiments using primary epithelial cells from a donor.

Next, we determined whether the down-regulation of TLR2 or MyD88 could affect cytokine secretion. To accomplish this, TNF and IL-8 levels were quantified from HGECs in which *TLR2* was silenced, after their subsequent stimulation with Poly I:C. As shown in **Figures 12A and 12B**, both TNF and IL-8 levels were significantly reduced to 70%. A similar Poly I:C treatment for silencing *MyD88* in HGECs also caused a significant reduction in TNF and IL-8 levels; this observation was in accordance with the interactions expected to occur between TLR2 and MyD88.

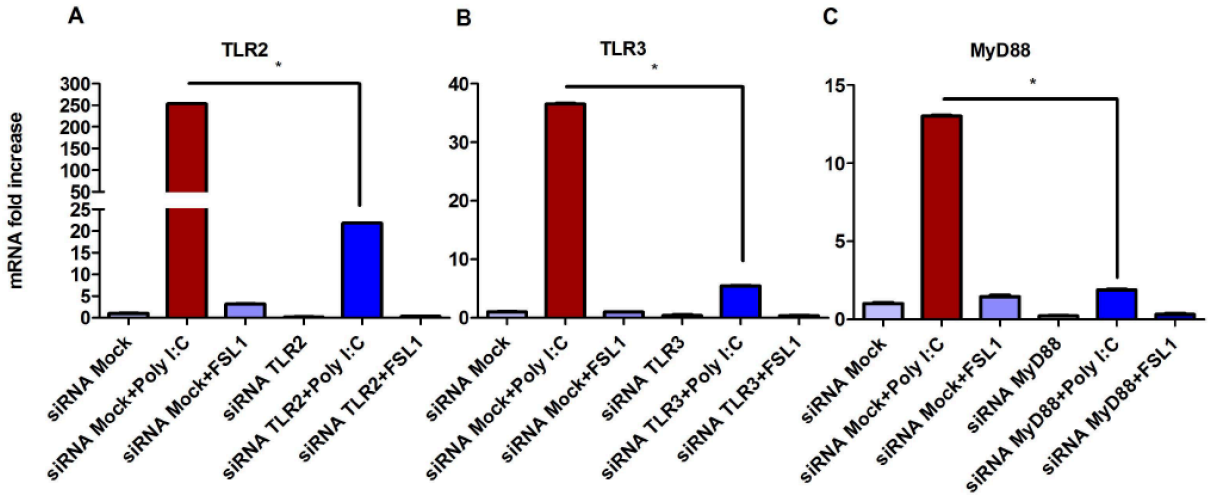

Figure 12: Poly I:C partially upregulates cytokine production by activating TLR2 and MyD88:

HGECs were stimulated with Poly I:C and FSL1 for 24 hours, after silencing *TLR2*, *TLR3*, and *MyD88*. ELISA results for supernatants showed that there was a significant decrease in IL-1beta (A), TNF (B) and IL-8 (C) levels, when *TLR3* and *MyD88* were silenced. Moreover, *TLR2* silencing significantly down-regulated the secretion of IL-1beta, TNF and IL-8 after poly I:C treatment. Striking differences were

observed when *Myd88* was silenced; IL-1beta, TNF, and IL-8 secretion levels were significantly down-regulated after poly I:C treatment. These data underline the unexpected role of Myd88 in the My88-independent pathway. Statistical test: We performed one-way ANOVA, followed by Tukey's multiple comparison test (*p < 0.05). Data represented as mean ±SE of three independent experiments using primary epithelial cells from a donor.

Activation of TLR2 by TLR3 is mediated via the induction of endogenous ligands HMGB1 and Hsp60.

Recent investigations into microbial molecules and their signaling pathways in host cells have identified several host-derived endogenous ligands(Al-Ofi and Al-Ghamdi 2018). These endogenous molecules include proteins that activate TLR signaling during pathological processes, even in the absence of microbial PAMPs(Singh, Biswas et al. 2016). These ligands include various types of molecules, such as proteins, fibronectin, heparin sulfate, biglycan, fibrinogen, oligosaccharides, and nucleic acids. Key proteins include high-mobility group box 1 (HMGB1), HSPs, tenascin-C, cardiac myosin, and S100 proteins, of which HMGB1 and Hsp60 have been specifically shown to activate the TLR2 signaling network(Chalmers, Eidelman et al. 2013). To understand the role of TLR2 in TLR3-mediated transcriptional activation more effectively, HGECs were stimulated with poly I:C and the TLR2 agonists Pam3CSK3 and FSL-1 for 4 and 24 hours, and the HMGB1 and Hsp60 expression levels were examined. Stimulation with poly I:C produced maximal levels of HMGB1 after 4 hours, while FSL-1 induced maximal levels of HMG1 after 24 hours **(Figure 13B).**

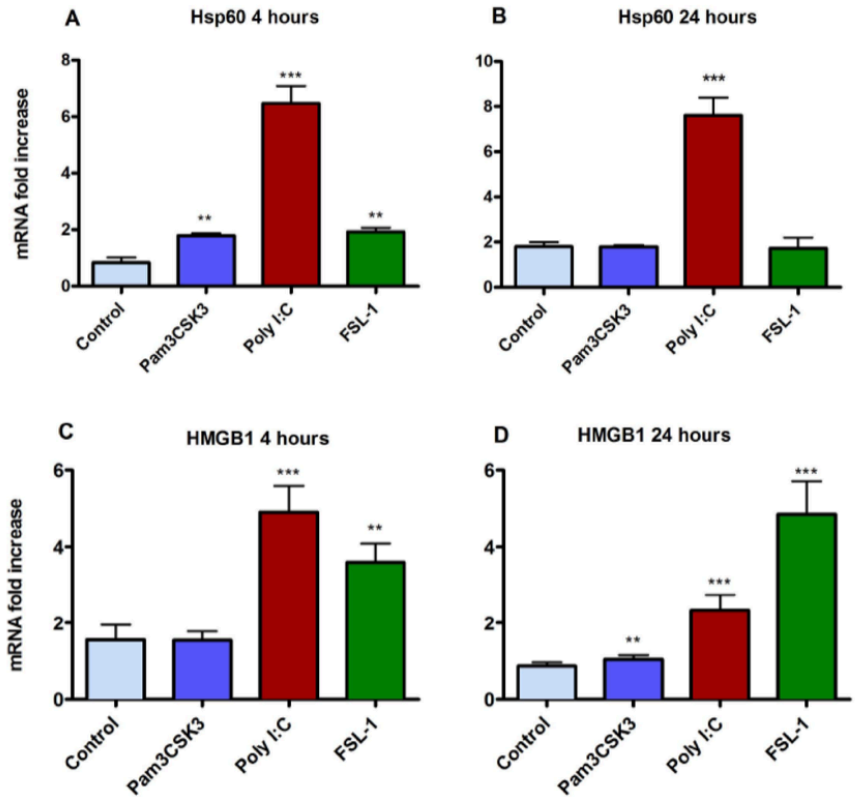

Figure 13: TLR3 stimulation activates endogenous TLR2 ligands:

HGECs are stimulated with Pam3CSK3, Poly I:C, and FSL-1 for 4 and 24 hours. The cDNA was used to measure HMGB1 and Hsp60 gene expression levels. Poly I:C increased the expression of both Hsp60 (HSPD1) and HMGB1 genes. Poly I:C treatment significantly increased the HMGB1 expression at the 4-hour time point (A), whereas FSL1 induced higher HMGB1 levels at 24 hours (B). On the other hand, the Hsp60 expression was minimally activated by FSL-1 treatment, but was robustly upregulated by Poly I:C at the 4 and 24 hour time points (C and D). Statistical test: We performed one-way ANOVA, followed by Tukey's multiple comparison test (*$p < 0.05$). Data represented as mean ±SE of three independent experiments using primary epithelial cells from a donor.

Additionally, poly I:C robustly promoted the expression levels of Hsp60 at both time points, while a minimal effect was observed after stimulation with FSL-1 **(Figures 13C and 13D)**. To check if the downregulation of TLR 2 occurred, we observed the expression levels of IL-8 and TNF. TNF and IL-8 levels were reduced by 95% when HMGB1 and HSP60 were silenced. When we treated epithelial cells with Poly I:C and silenced both endogenous ligands Hsp60 and HMGB1, the TNF and IL-8 expression levels were downregulated, in comparison to levels observed after media alone was treated with Poly I:C and SiRNA was mock treated with Poly I:C. This shows the role of HMGB1 and HSP60 in the functional activation of TLR2 via Hsp60 and HMGB1 **(Figure 14).**

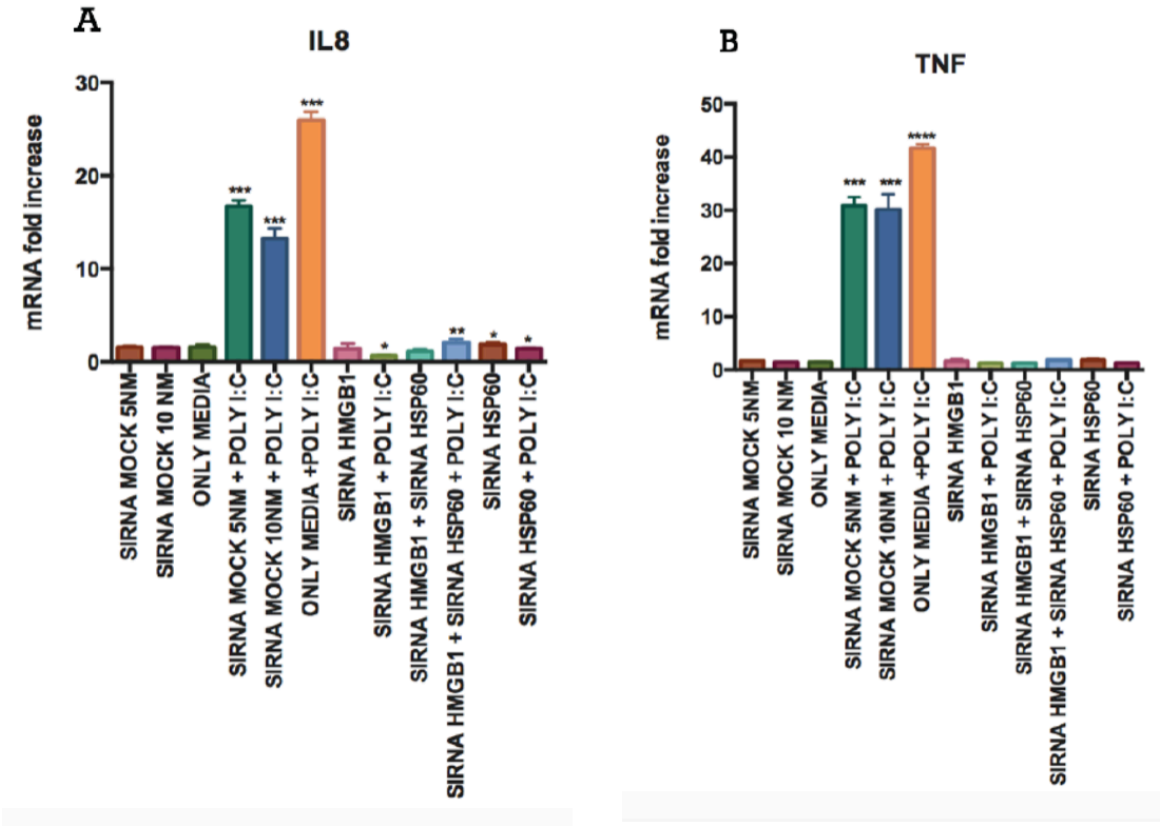

Figure 14: HMGB1 and Hsp60 knockouts reduce pro-inflammatory cytokine levels when epithelial cells are stimulated with Poly IC:

HGECs stimulated with Poly I:C for 4 and 24 hours. Poly I:C treatment increased the expression of both *IL-8* and *TNF*. On the other hand, the knockout of *Hsp60* and *HMGB1* robustly reduce the expression of TNF and IL-8 after treatment with Poly I:C at the 4 and 24 hour time points. Statistical test: We performed one-way ANOVA, followed by Tukey's multiple comparison test (*$p < 0.05$). Data represented as mean ±SE of three independent experiments using primary epithelial cells from a donor.

A western blot was performed and the results showed that the knockout of only HMGB1 reduces the protein Hsp60 level, which reveals a co-interaction between these two molecules **(Figure 15).**

Together, our data demonstrate that TLR3 stimulation not only leads to TLR2 up-regulation, but also induces the production of endogenous ligands of TLR2 that might be responsible for the activation of pro-inflammatory cytokines **(Figure 16).**

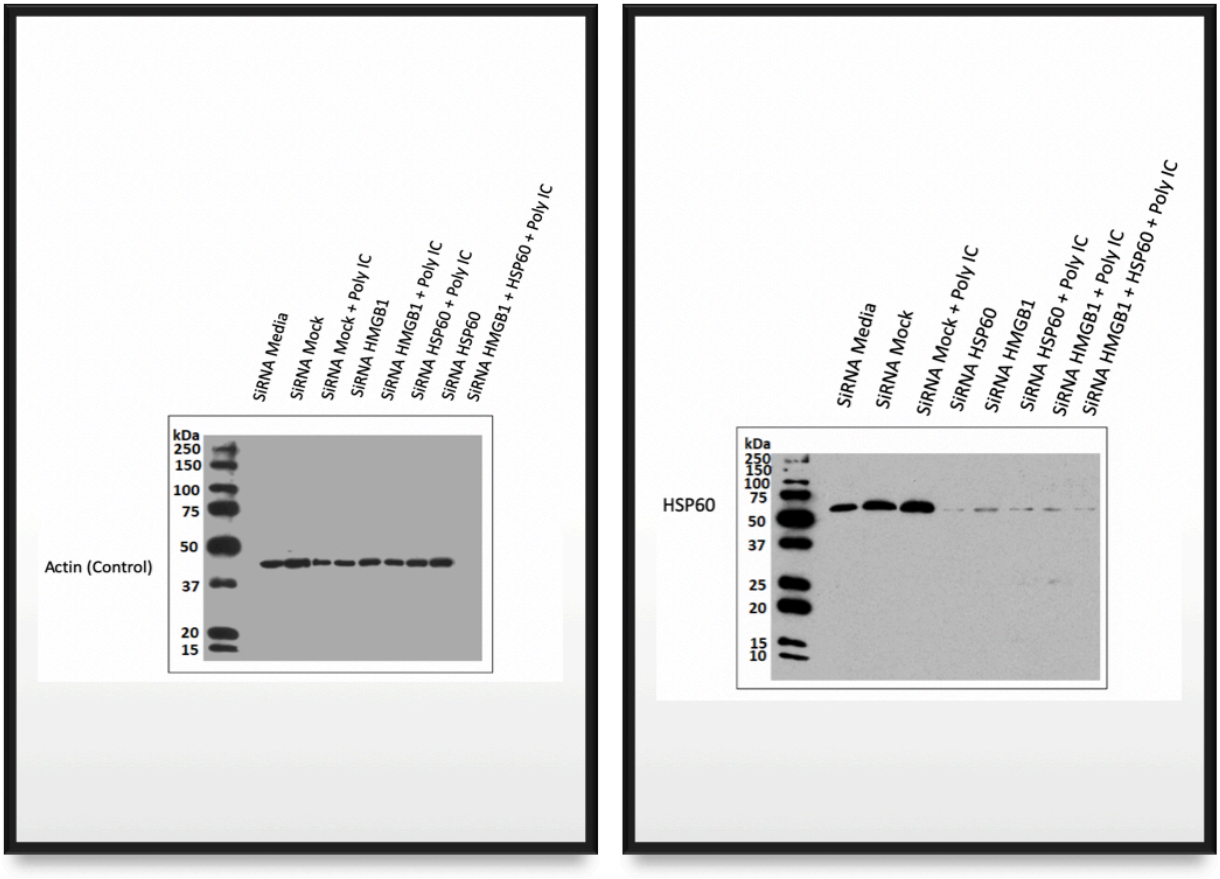

Figure 15 : Immunoblot from epithelial cells treated with 10 ng of siRNA HMGB1 and Hsp60 challenged with Poly I:C:

Cells in which HMGB1 was knocked out caused a significant reduction in Hsp60 (60 kDa) expression levels, as compared to that observed in control cells. HMGB1 and Hsp60 seem to work as a complex. Levels of the housekeeping protein β-Actin were similar between samples.

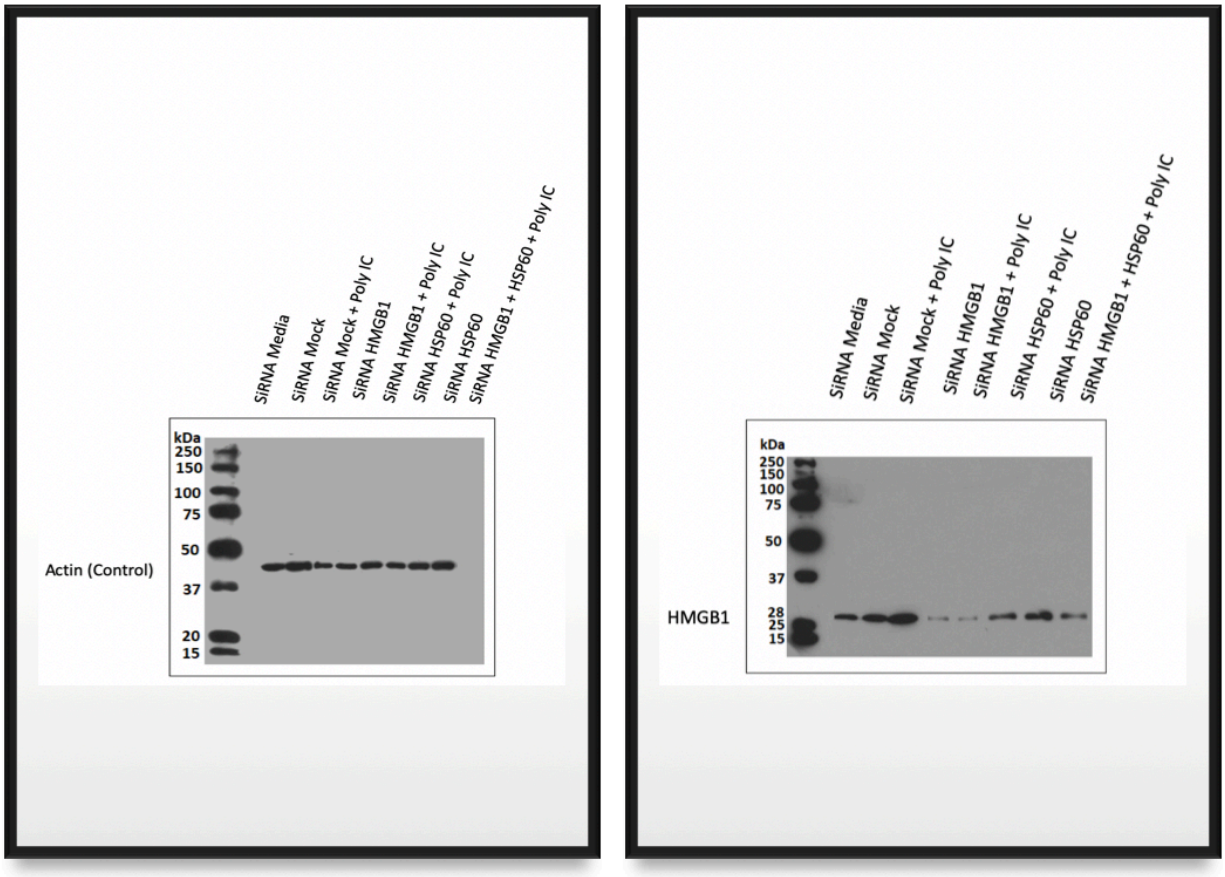

Figure 16 : Immunoblot from epithelial cells treated with 10 ng of siRNA HMGB1 and Hsp60 challenged with Poly IC:

Cells in which HMGB1 was knocked out caused a significant reduction in Hsp60 (60 kDa) expression levels, as compared to that observed in control cells. Levels of the housekeeping protein β-Actin were similar between samples.

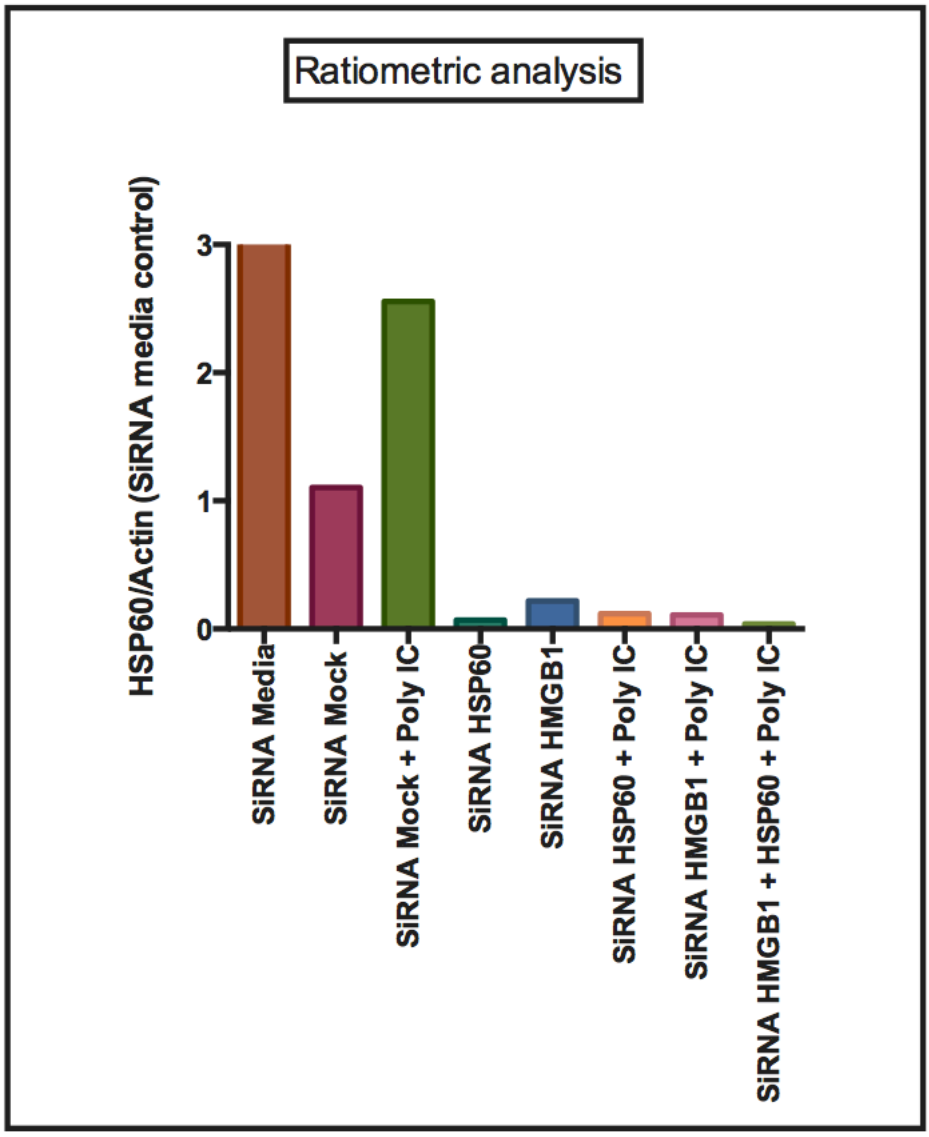

Figure 17 : Ratiometric analysis of Immunoblot from epithelial cells treated with 10 ng of siRNA HMGB1 and Hsp60 challenged with Poly I:C:

Cells in which HMGB1 was knocked out caused a significant reduction in Hsp60 (60 kDa) expression levels, as compared to that observed in control cells. Levels of the housekeeping protein β-Actin were similar between samples

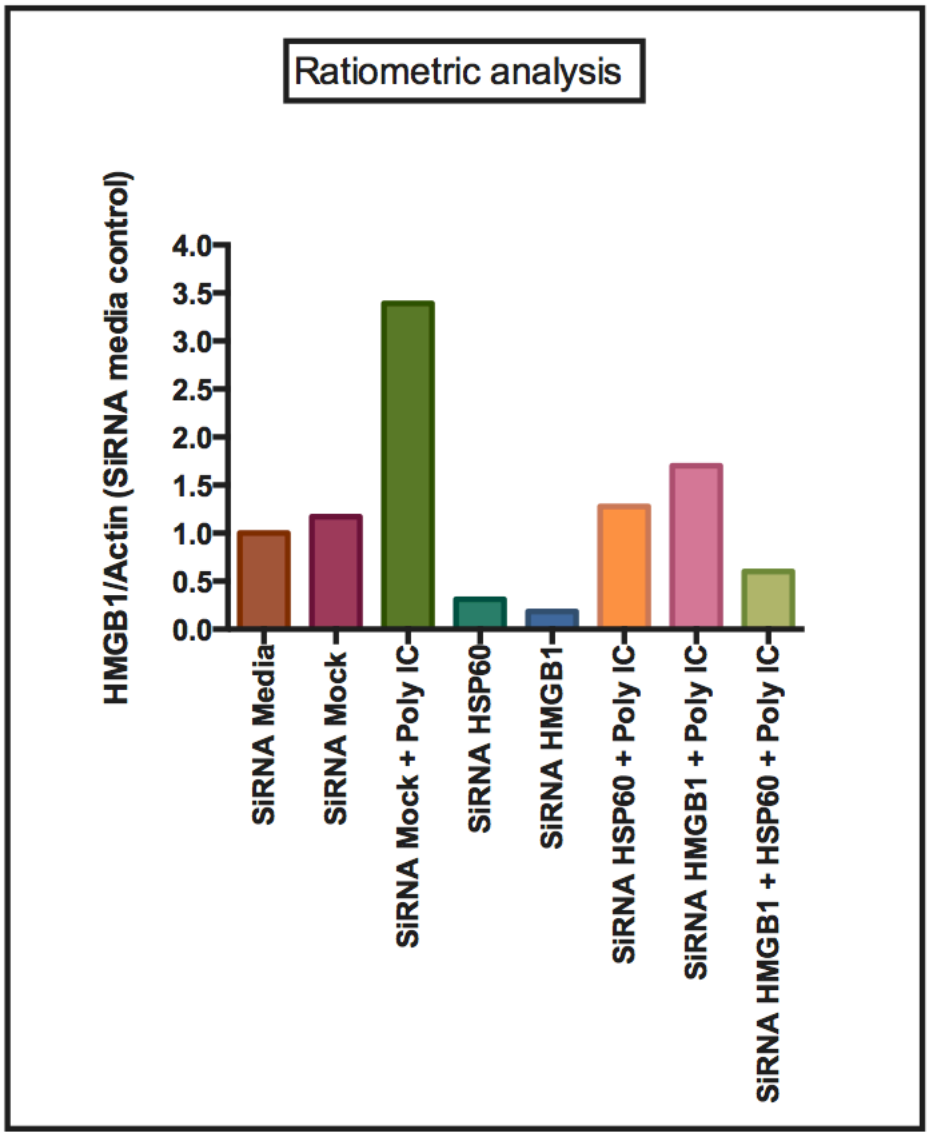

Figure 18 : Ratiometric analysis of Immunoblot from epithelial cells treated with 10 ng of siRNA HSP60 and HMGB1 challenged with Poly I:C:

Cells in which HMGB1 was knocked out caused a significant reduction in Hsp60 (60 kDa) expression levels, as compared to that observed in control cells. Levels of the housekeeping protein β-Actin were similar between samples.

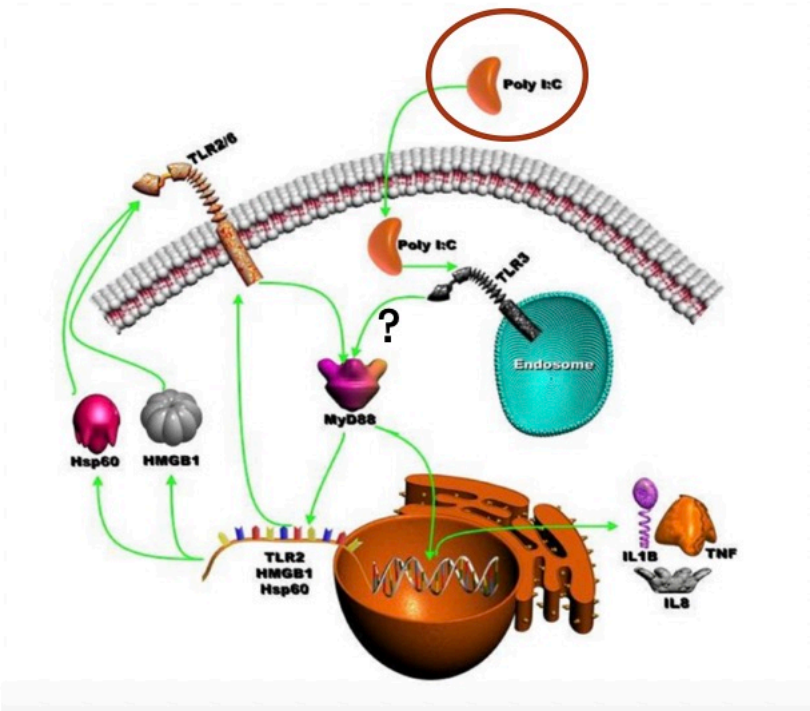

Figure 19: Diagram showing the role of TLR3 in endogenous ligand activation and triggering of the TLR2 receptor:

The stimulation of TLR3 by Poly I:C leads not only to TLR2 up-regulation, but also induces the production of endogenous ligands of TLR2 that might be responsible for the activation of pro-inflammatory cytokines.

Lower expression of the pro-inflammatory gene TNF and GRO and transcription factors NF-κB and IRF-3 in a Myd88$^{-/-}$ mouse

The expression of pro-inflammatory transcriptional factors and cytokines was successfully reduced after epithelial cells of Myd88$^{-/-}$ mice were stained, after their skin was treated with Poly I:C.

Immunopositive cells were a majority on the epithelial layer of wild type mice compared to Myd88$^{-/-}$ mice group. We also have encountered few immunopositive cells on the connective tissue. That is expected as the connective tissue have fibroblasts and immune cells which also present TLR3 in their endosome organelles. TNF wide type group showed 90% of positive cells compared to 35% in TNF Myd88$^{-/-}$ mice group. GRO wide type group showed 75% of positive cells compared to 30% in GRO Myd88$^{-/-}$ mice group.

NFkB wide type group showed 95% of positive cells compared to 40% in NFkB Myd88$^{-/-}$ mice group. IRF wide type group showed 80% of positive cells compared to 35 % in IRF-3 Myd88$^{-/-}$ mice group.

When we compared Isotype antibody and control (mouse treated with vehicle and not Poly I:C) We found Isotype and control to be the same **Figure 20**.

All images for each specific anti-body were capturing using the same capture time.TNF and GRO capture time was 10s using the Z-stacking technology. NFkb and IRF capture time images were 15 s with Z-stacking technology.

The statistical differences in the mean immunofluorescence intensity values for NF-κB, IRF-3, and TNF were significant in wild type mice challenged with Poly I:C, as compared to the values for Myd88 knockout mice challenged with Poly I:C **(Figure 33).**

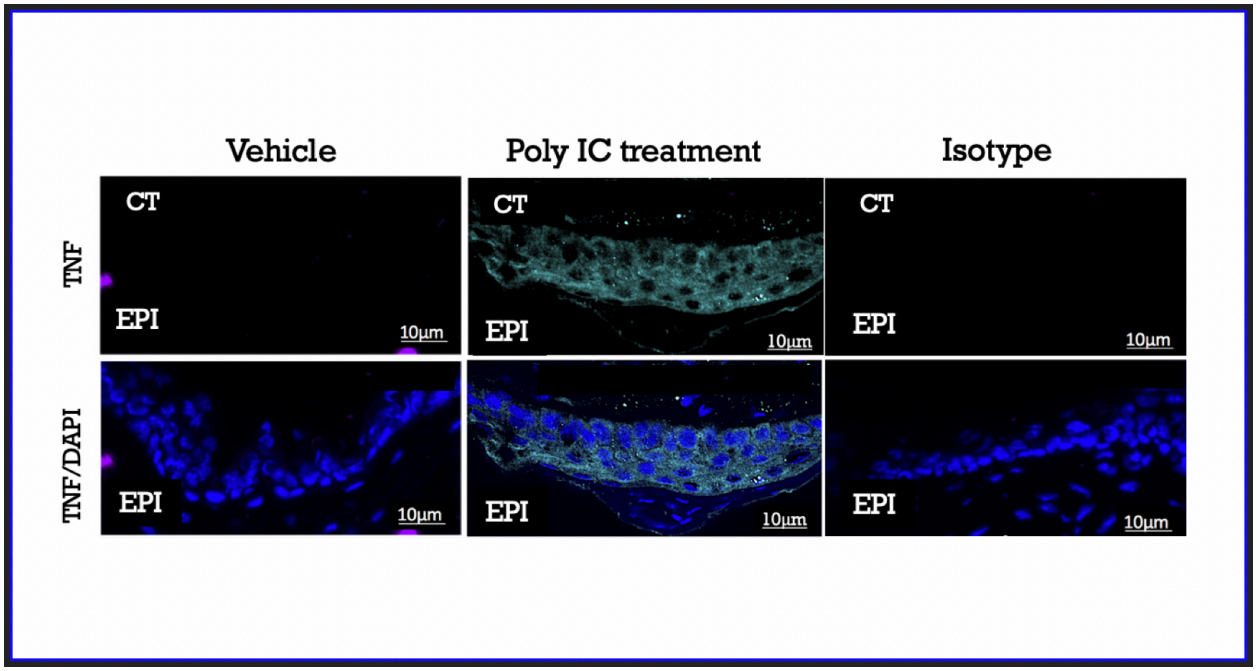

Figure 20 : Epithelial cells staining for the visualization of TNF cytokines in wild type mice

Epithelial cells challenged with Poly I:C. Poly I:C treatment increase TNF secretion within the cytoplasm compared to Vehicle.(Original magnification 60x). EPI, epithelium; C, connective tissue. N = 8 mice per group. Bar = 10 µm.

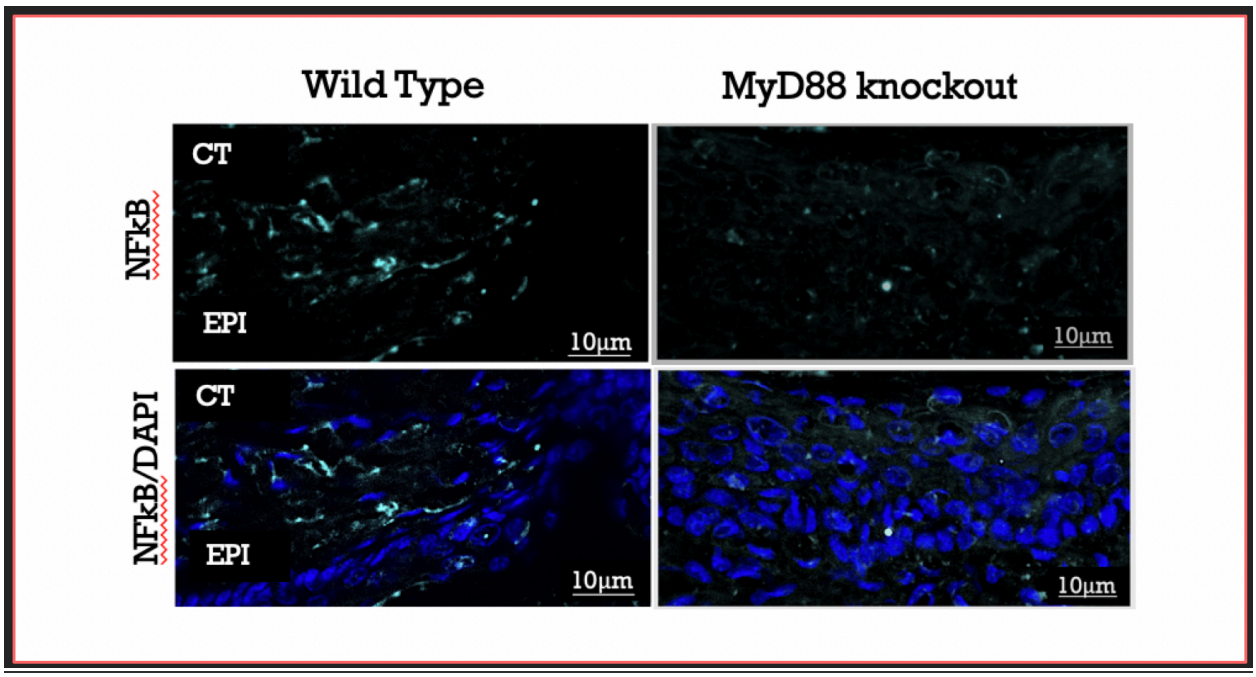

Figure 21: Epithelial cell staining for the visualization of phosphorylated NF-κB transcriptional factors in wild type mice versus MyD88 knockout mice.

Epithelial cells from wild type mice and MyD88 knockout mice challenged with Poly I:C. Immunofluorescence was observed because of the P-NF-kappaB p65 (S536) in skin tissue sections treated with Poly I:C. A higher mean of fluorescence intensity was observed on the surface skin of the wild type mouse (green [P-NF-kappaB p65]; blue [DAPI]).(Original magnification 60x). EPI, epithelium;CT, connective tissue. N = 8 mice per group. Bar = 10 µm.

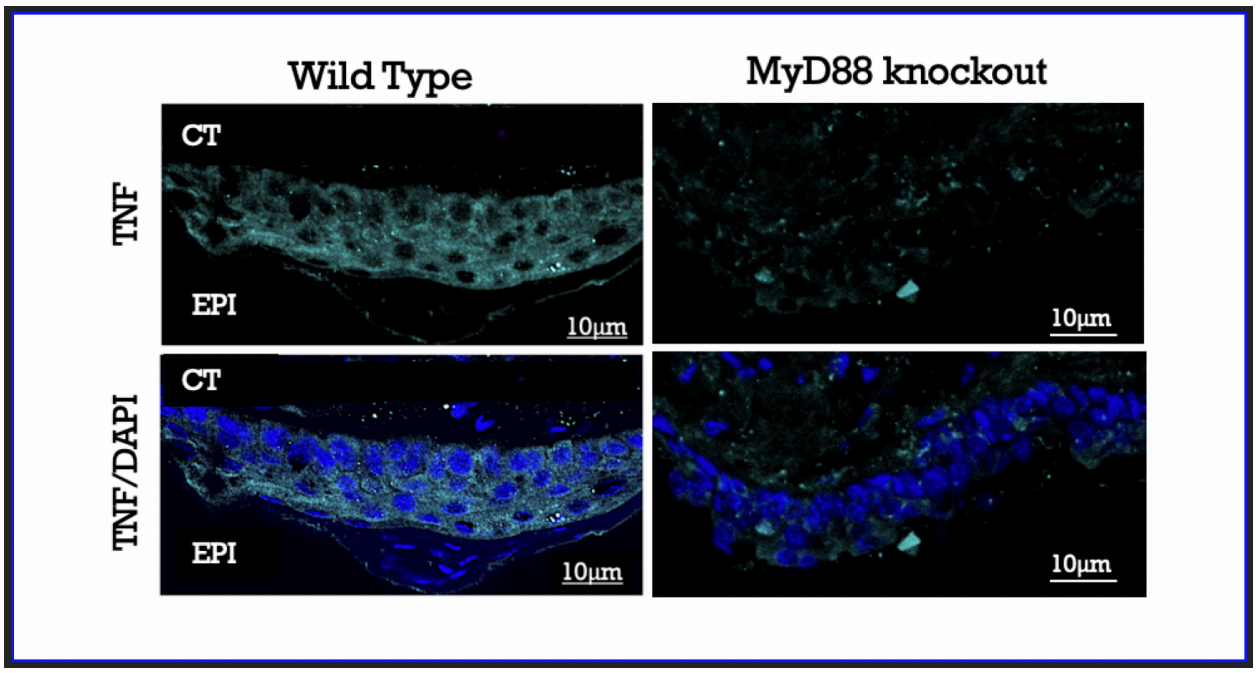

Figure 22: Epithelial cells staining for the visualization of TNF cytokines in wild type mice versus MyD88 knockout mice:

Epithelial cells from wild type mice and MyD88 knockout mice challenged with Poly I:C: Immunofluorescence was observed in skin tissue sections treated with Poly I:C enabled the visualization of TNF. A higher mean fluorescence intensity was observed on the skin surface of the wild type mouse (green [TNF]; blue [DAPI]). (Original magnification 60x). EPI, epithelium;CT, connective tissue. N = 8 mice per group. Bar = 10 µm.

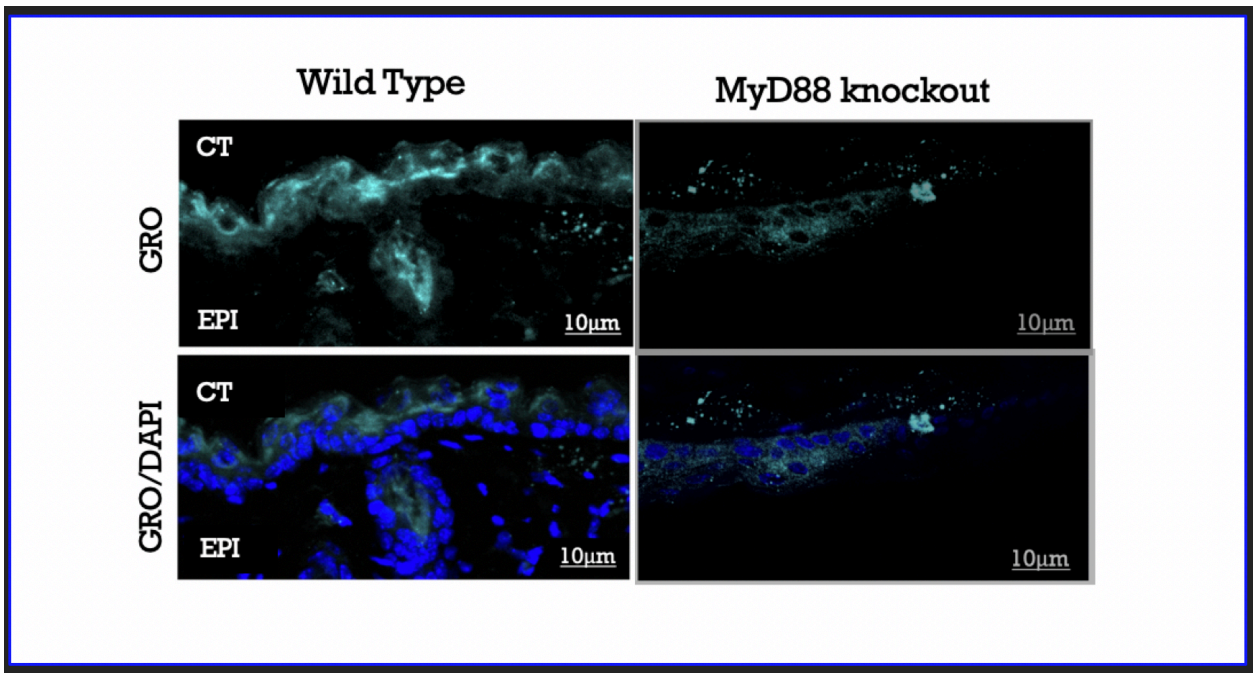

Figure 23: Epithelial cells staining for the visualization of GRO cytokines in wild type mice versus MyD88 Knockout mice:

Epithelial cells from wild type mice and MyD88 knockout mice challenged with Poly I:C: Immunofluorescence was observed in skin tissue sections treated with Poly I:C enabled the visualization of GRO. A higher mean fluorescence intensity was observed on the skin surface of the wild type mouse (green [GRO]; blue [DAPI]). Original magnification 60x). EPI, epithelium;CT, connective tissue. N = 8 mice per group. Bar = 10 µm.

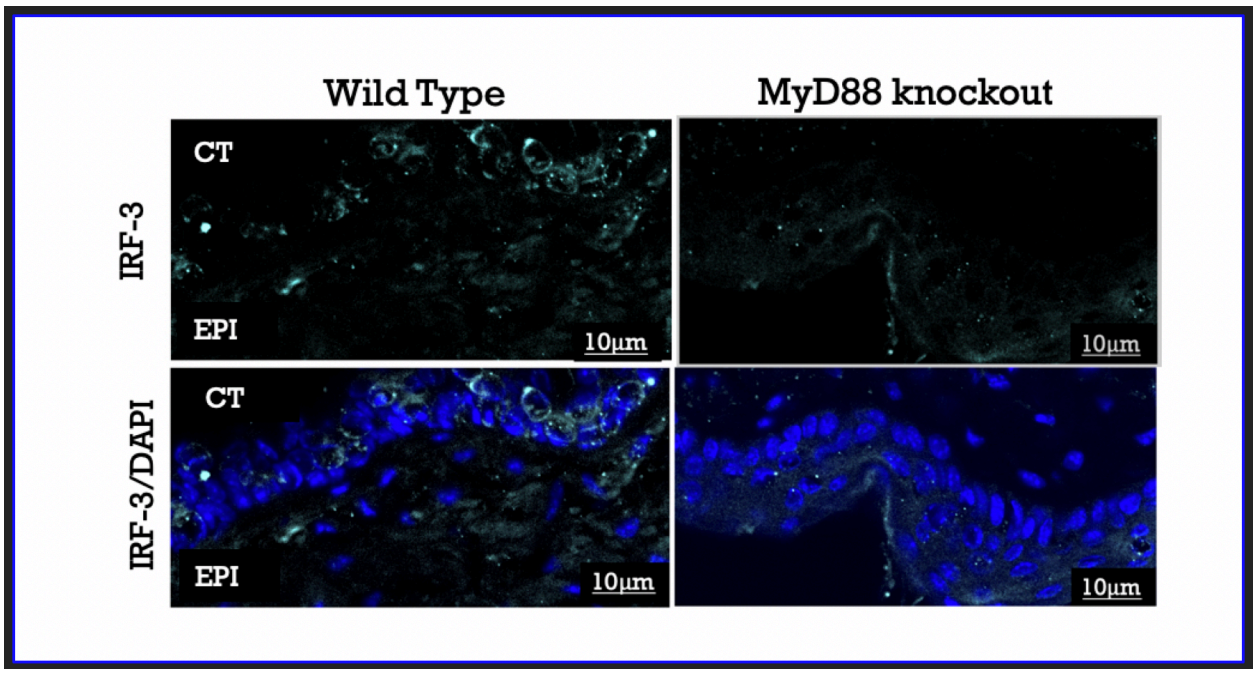

Figure 24: Epithelial cell staining for the visualization of IRF-3 factor in wild type mice versus MyD88 knockout mice.

Epithelial cells from wild type mice and MyD88 knockout mice challenged with Poly I:C: Immunofluorescence was observed because of the use of p-IRF-3(S536) in skin tissue sections treated with Poly I:C. A higher mean of fluorescence intensity was observed on the surface skin of the wild type mouse (green [IRF-3]; blue [DAPI]). Original magnification 60x). EPI, epithelium; CT, connective tissue. N = 8 mice per group. Bar = 10 µm.

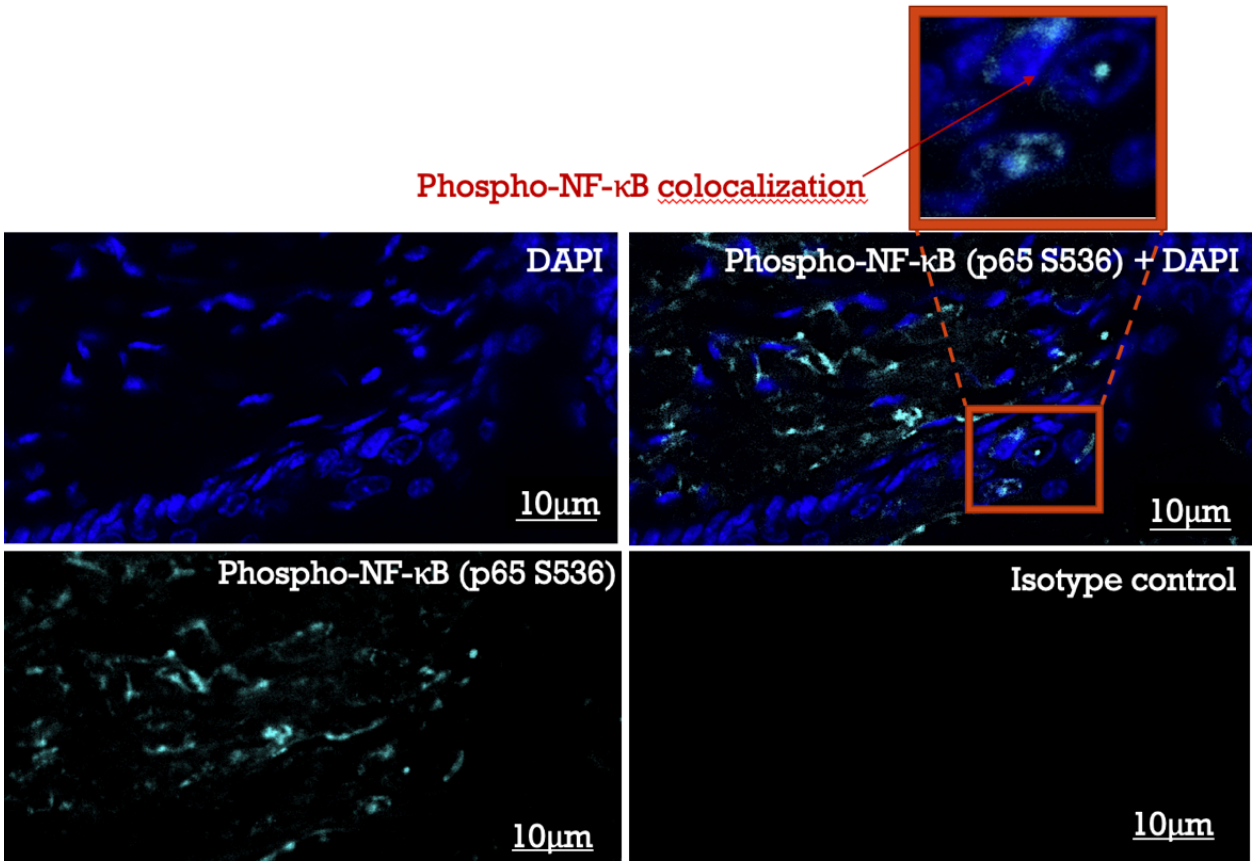

Figure 25: Epithelial cell staining for the visualization of phosphorylated NF-κB transcriptional factors in wild type mice.

Epithelial cells from wild type mice challenged with Poly I:C: Immunofluorescence was observed because of the P-NF-kappaB p65 (S536) in skin tissue sections treated with Poly I:C. A higher mean of fluorescence intensity was observed on the surface skin of the wild type mouse (green [P-NF-kappaB p65]; blue [DAPI]). Red box: Phospho-NFkB colocalization. Original magnification 60x). EPI, epithelium; CT, connective tissue. N = 8 mice per group. Bar = 10 µm.

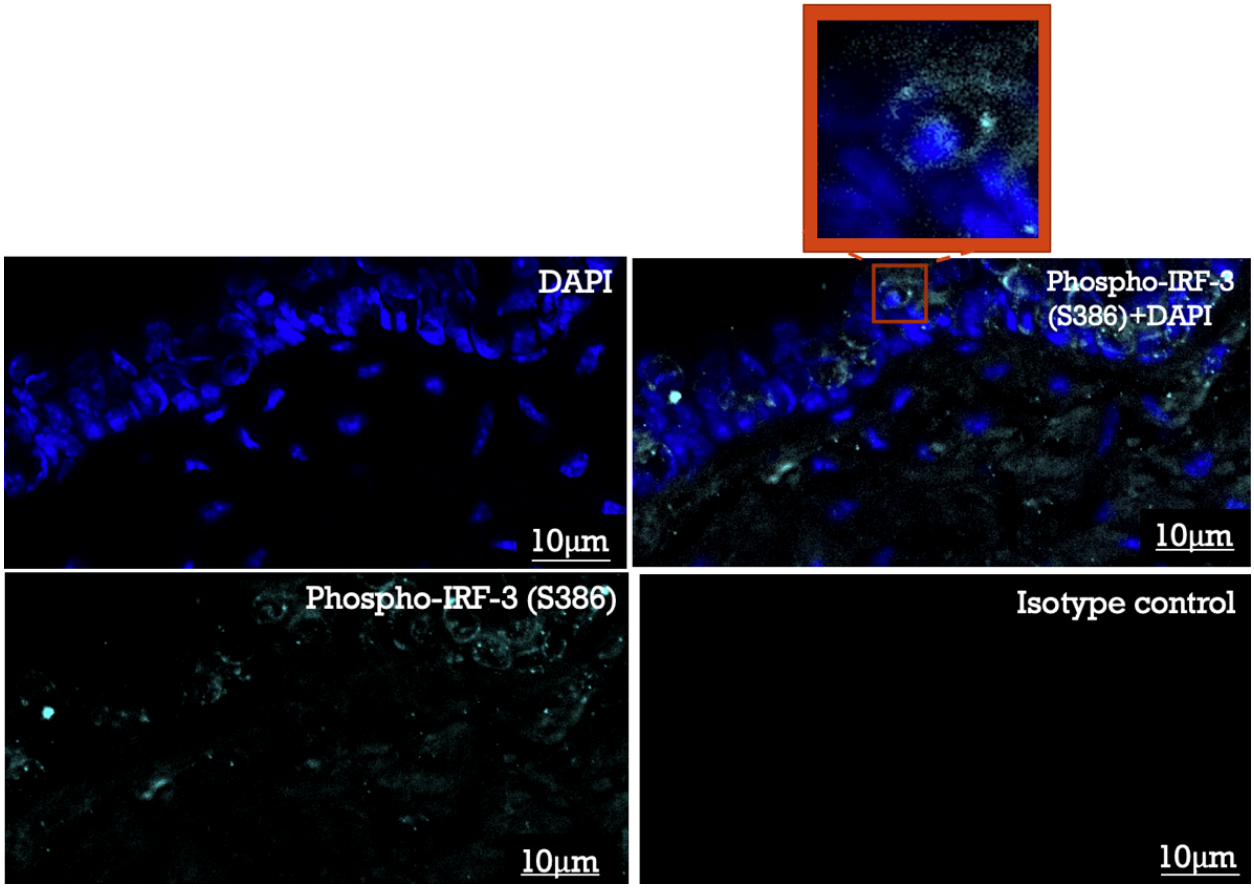

Figure 26: Epithelial cell staining for the visualization of IRF-3 factor in wild type mice.

Epithelial cells from wild type mice challenged with Poly I:C: Immunofluorescence was observed because of the use of p-IRF-3(S536) in skin tissue sections treated with Poly I:C. A higher mean of fluorescence intensity was observed on the surface skin of the wild type mouse (green [IRF-3]; blue [DAPI]). Red box: IRF-3 colocalization. Original magnification 60x). EPI, epithelium; CT, connective tissue. N = 8 mice per group. Bar = 10 µm.

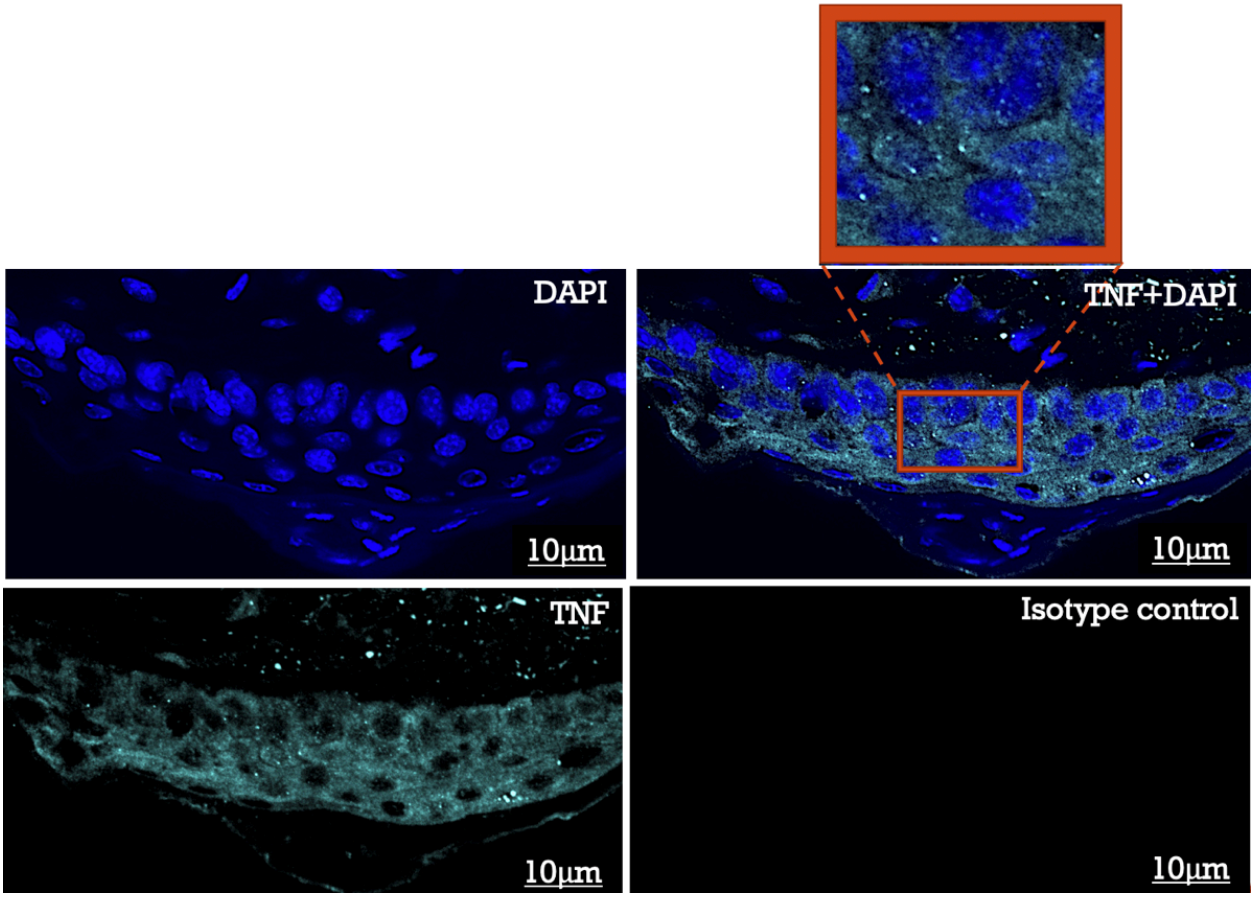

Figure 27: Epithelial cells staining for the visualization of TNF cytokines in wild type mice:

Epithelial cells from wild type mice challenged with Poly I:C: Immunofluorescence was observed in skin tissue sections treated with Poly I:C enabled the visualization of TNF. A higher mean fluorescence intensity was observed on the skin surface of the wild type mouse (pink [TNF]; blue [DAPI]). Red box: TNF secretion within the cytoplasm. Original magnification 60x). EPI, epithelium; CT, connective tissue. N = 8 mice per group. Bar = 10 µm.

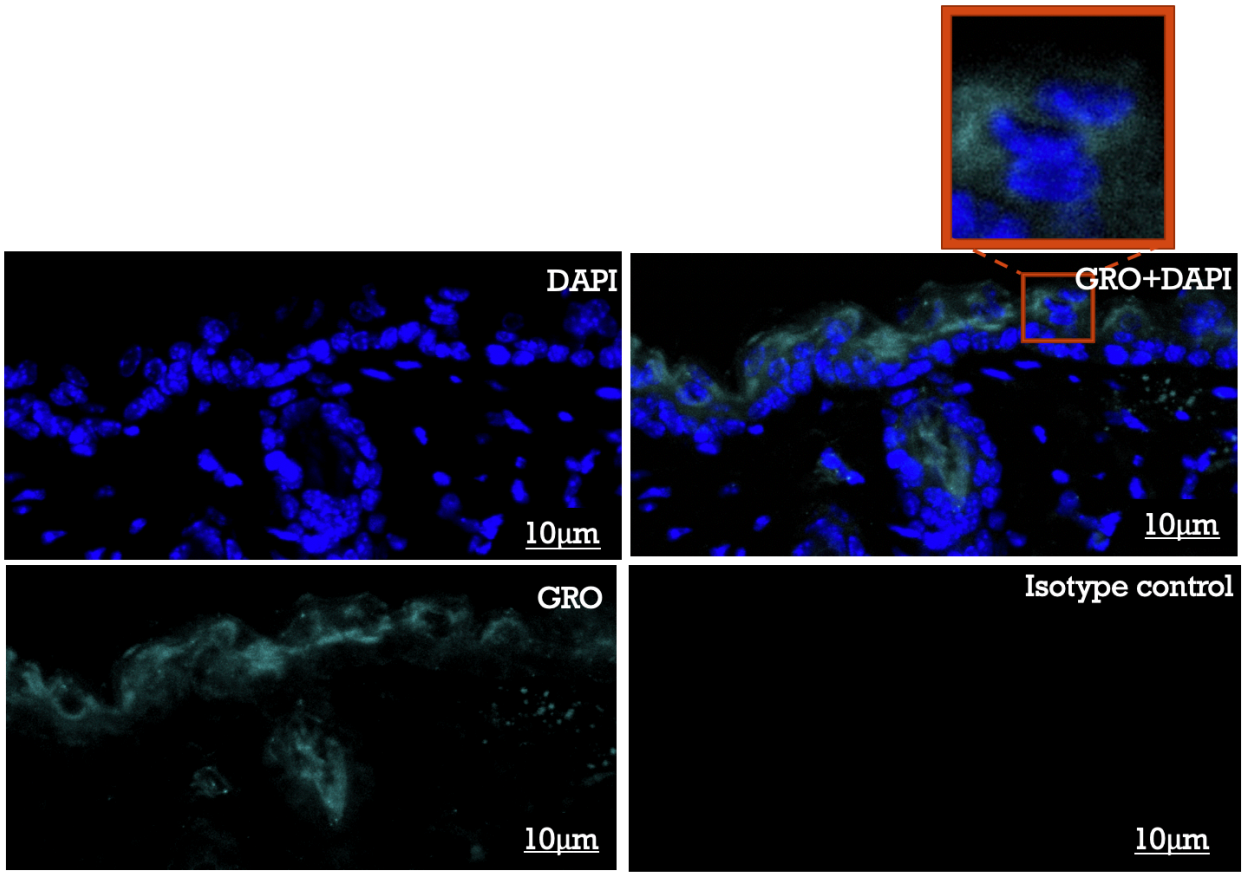

Figure 28: Epithelial cells staining for the visualization of GRO cytokines in wild type mice:

Epithelial cells from wild type mice challenged with Poly I:C: Immunofluorescence was observed in skin tissue sections treated with Poly I:C enabled the visualization of GRO. A higher mean fluorescence intensity was observed on the skin surface of the wild type mouse (pink [GRO]; blue [DAPI]). Red box: GRO secretion within the cytoplasm. Original magnification 60x). EPI, epithelium; CT, connective tissue. N = 8 mice per group. Bar = 10 µm.

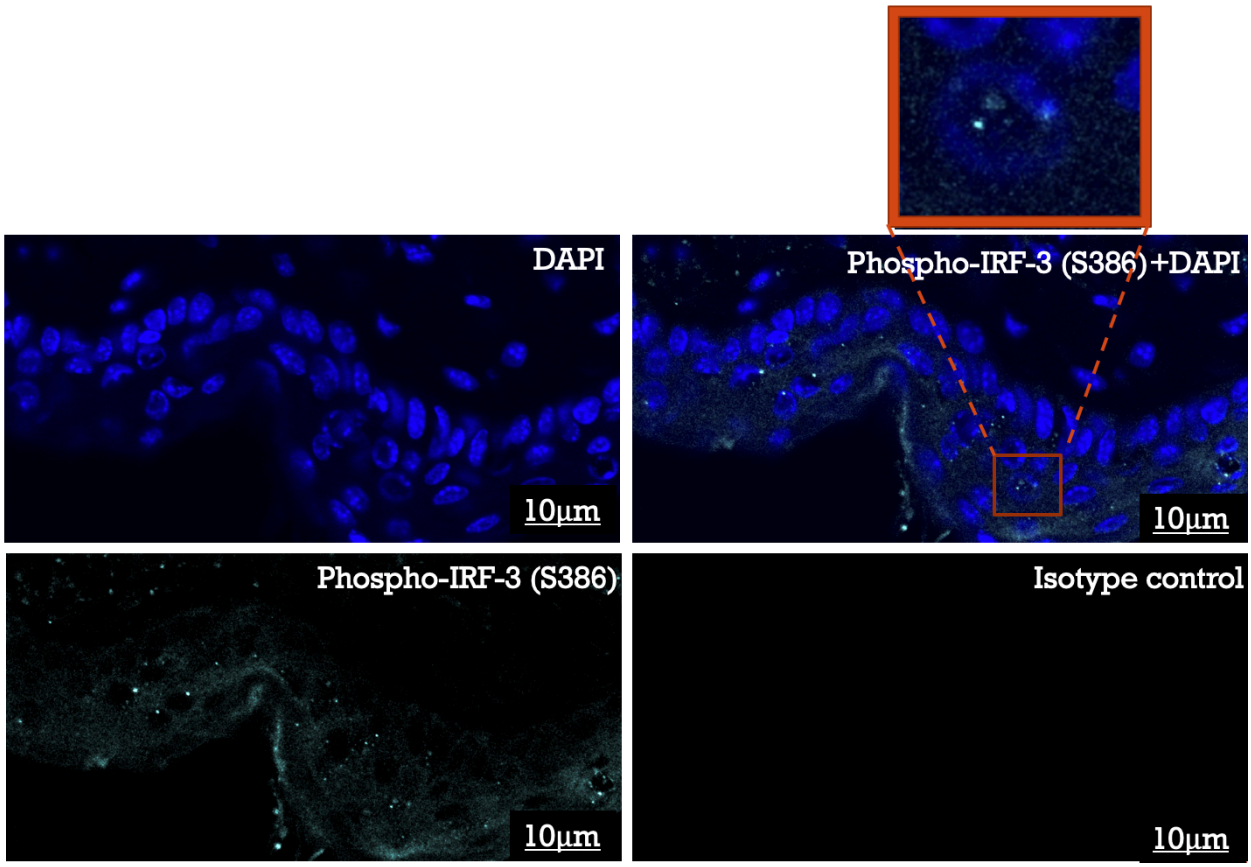

Figure 29: Epithelial cell staining for the visualization of IRF-3 transcriptional factor in MyD88 knockout mice:

Epithelial cells from MyD88 knockout mice challenged with Poly I:C: Immunofluorescence was observed because of the use of p-IRF-3(S536) in skin tissue sections that received Poly I:C treatment. A low mean fluorescence intensity was observed on the skin surface of the treated MyD88 knockout type mouse (green [IRF-3]; blue [DAPI]). Red box: IRF-3 colocalization. Original magnification 60x). EPI, epithelium; CT, connective tissue. N = 8 mice per group. Bar = 10 µm.

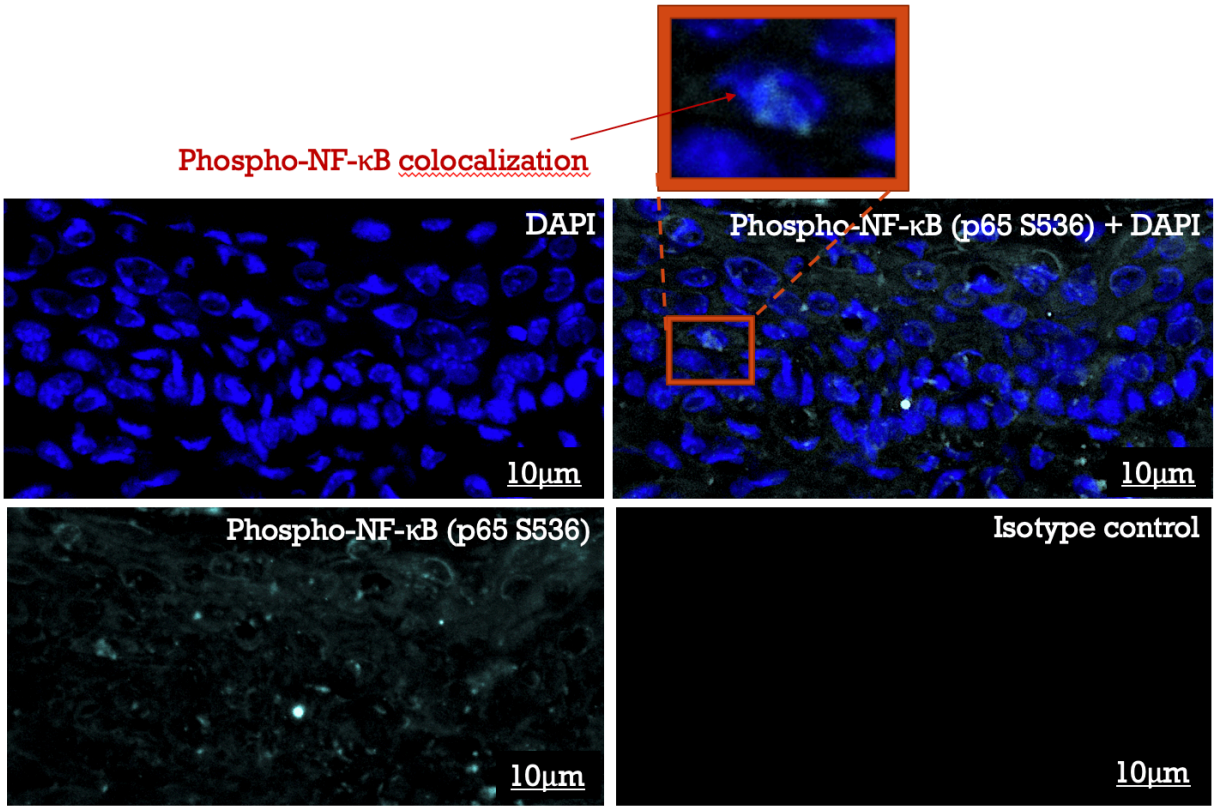

Figure 30: Epithelial cell staining for the visualization of phosphorylated NF-κB transcriptional factor in MyD88 knockout mice:

Epithelial cells from MyD88 knockout mice challenged with Poly I:C: Immunofluorescence was observed because of the use of P-NF-kappaB p65 (S536) in skin tissue sections that received Poly I:C treatment. A low mean fluorescence intensity was observed on the skin surface of the treated MyD88 knockout type mouse (green [P-NF-kappaB p65]; blue [DAPI]). Red box: P-NF-kappaB p65 colocalization. Original magnification 60x). EPI, epithelium; CT, connective tissue. N = 8 mice per group. Bar = 10 µm.

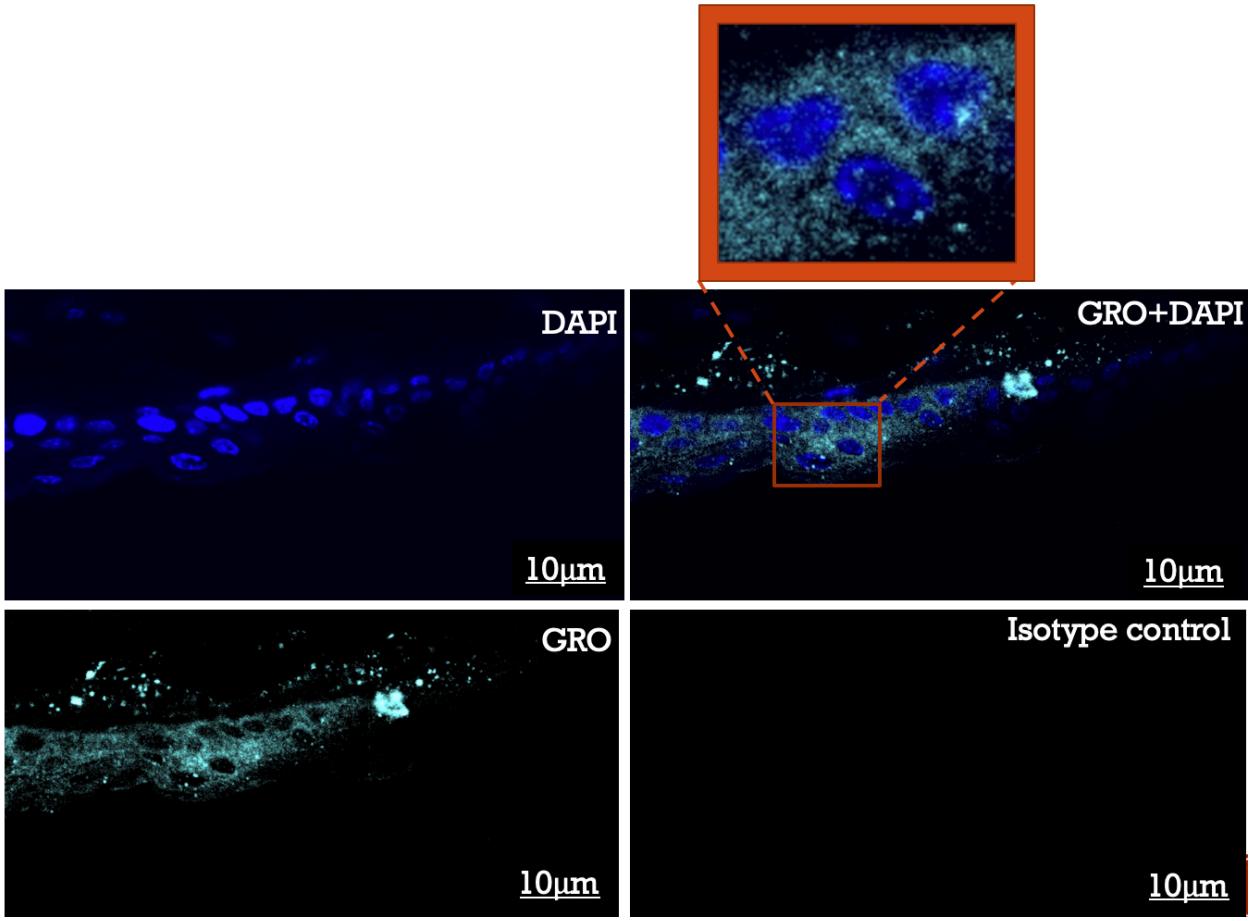

Figure 31: Epithelial cell staining for the visualization of GRO cytokines in MyD88 knockout mice.

Epithelial cells from MyD88 knockout mice challenged with Poly I:C: Immunofluorescence was observed because of the use of GRO anti-body in the skin tissue sections after Poly I:C treatment: low mean of fluorescence intensity was noted on the surface skin of MyD88 knockout type mouse that received Poly I:C treatment (green [GRO]; blue [DAPI]). Red box: GRO secretion within the cytoplasm. Original magnification 60x). EPI, epithelium; CT, connective tissue. N = 8 mice per group. Bar = 10 µm.

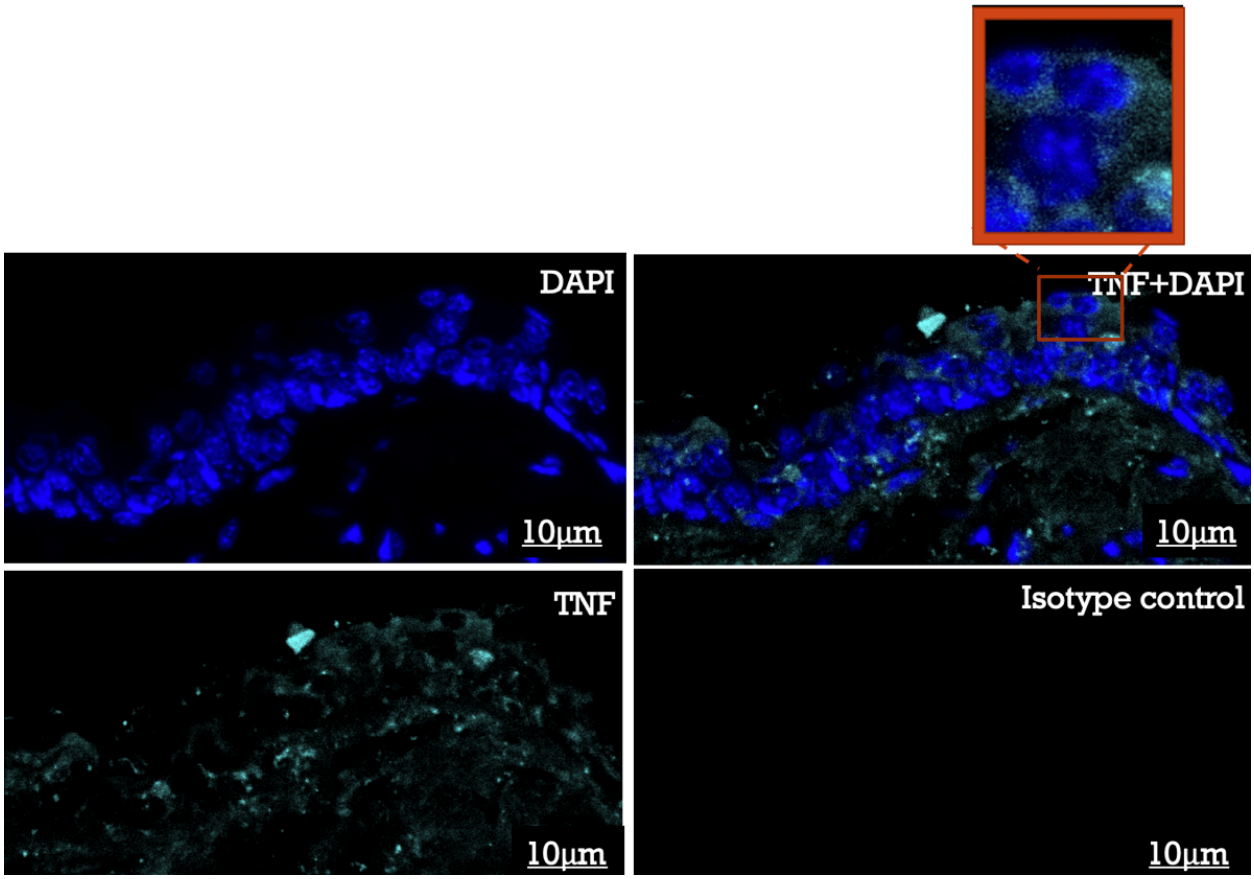

Figure 32: **Epithelial cell staining for the visualization of TNF cytokines in MyD88 knockout mice.**

Epithelial cells from MyD88 knockout mice challenged with Poly I:C: Immunofluorescence was observed because of the use of TNF anti-body in the skin tissue sections that received Poly I:C treatment. A low mean fluorescence intensity was noted on the skin surface of the treated MyD88 knockout mouse (green [TNF]; blue [DAPI]). Red box: TNF secretion within the cytoplasm. Original magnification 60x). EPI, epithelium; CT, connective tissue. N = 8 mice per group. Bar = 10 µm.

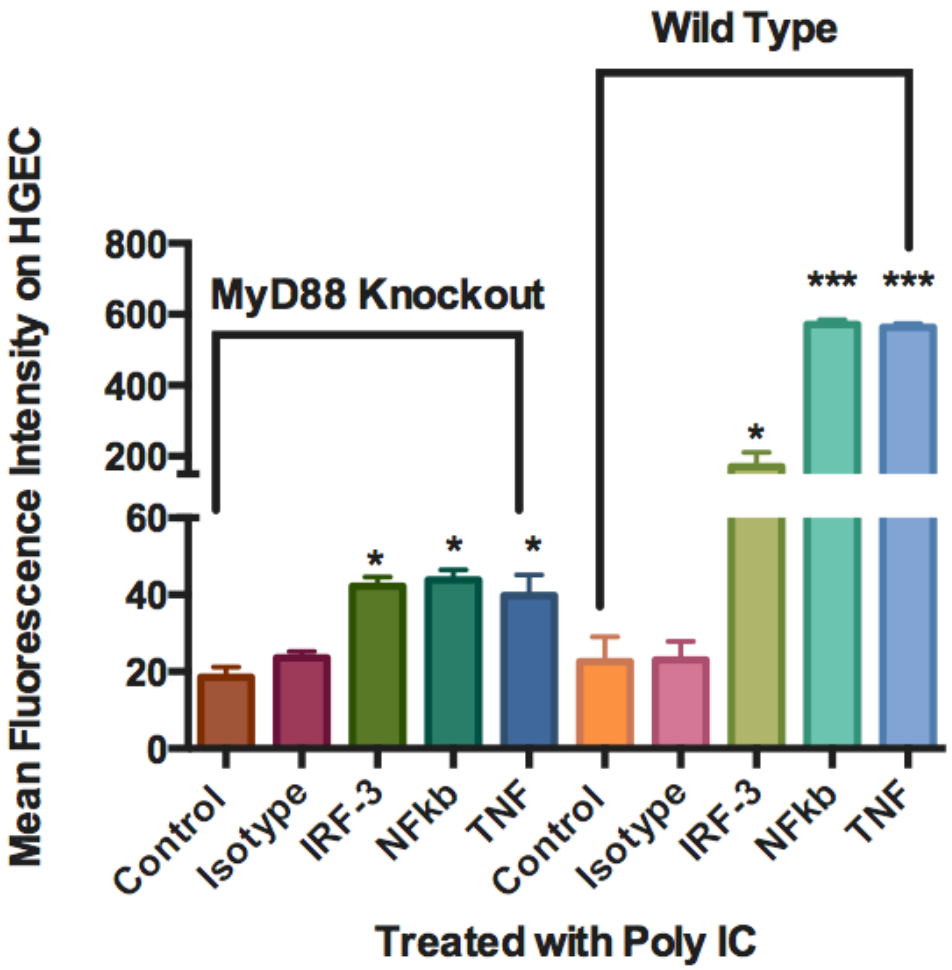

Figure 33: The intensity of immunofluorescence within the epidermis, measured using Image J software:

Epithelial cells from wild type mice and MyD88 knockout mice challenged with Poly I:C. Immunofluorescence of IRF-3, NF-κB transcriptional factors, and TNF cytokines in the epithelial cells of mouse skin sections: The mean fluorescence intensity is higher in the stained cells of the wild type mouse, compared to the values for the knockout mouse. Statistical test: We performed one-way ANOVA, followed by Tukey's multiple comparison test (*$p < 0.05$). Results represent mean ± SE values

Chapter 5: Discussion and Future Direction

The binding of ligands to TLRs induces downstream signaling via two distinct pathways, i.e. the MyD88-dependent and TRIF-dependent pathways, for the induction of pro-inflammatory cytokines and IFN genes (Bagchi, Herrup et al. 2007, Kenny, Talbot et al. 2009). However, MyD88 is used by TLR2, TLR5, TLR7, TLR8, and TLR9, while TRIF is used by TLR3. TLR4 uses both MyD88- and TRIF-dependent mechanisms (Barton and Medzhitov 2002, Sahoo, Basu et al. 2013). However, the involvement of TLR3 with the MyD88 signaling network remains controversial. While Takumi, Kawasaki at al. 2014 advocates that TLR3 signaling pathway is totally independent of MYD88 other studies (Jingya, Xia at al. 2013) indicate that both TLR3- and MyD88-dependent signaling play important roles in shaping the development of humoral responses to the single-cycle vaccine RepliVax WN (Jingya, Xia at al. 2013). Our study corroborates with Jingya, Xia studies showing that Poly I:C up-regulated TLR2 levels in siTLR2 treated cells; this suggests that TLR3 stimulation leads to a strong transcriptional activation of TLR2. Furthermore, we showed that Poly I:C causes an increase in MYD88 expression, even though it is thought to be independent.

TLR3 is identified as a major MyD88-independent PRR (pattern-recognition receptor) for the induction of type-1 IFN, in response to different viral infections. However, the role of TLR3 in bacterial infections is poorly understood (Angeliki, Xagorari at al. 2008). MyD88 is an important adapter molecule that recognizes all TLR ligands except TLR3 (Xia, Winkelmann et al. 2013). Our data demonstrate the interactions between Myd88 in the regulation of TLR2 expression by TLR3, which suggests that Myd88 expression was

stimulated indirectly. In addition, it was shown for the first time that the up-regulation of TLR2 occurred in Poly I:C treated cells, as *TLR2* silencing significantly down-regulated IL-8 and TNF secretion after Poly I:C treatment. After *Mydd88* was silenced, the secretion of both TNF and IL-8 was significantly down-regulated upon Poly I:C treatment. The data emphasize the unexpected role of MyD88 in the MyD88-independent pathway.

The recognition of pathogens by epithelial cells involves interplay between PAMPs (pathogen-associated molecular patterns) and various host PRRs. The occurrence of this interaction during infection results in the release of various inflammatory mediators and chemokines that induce an influx of neutrophils into the site of infection, activates nearby macrophage and antigen presenting cells, and ultimately decides the acquired immune response (Pan, Fisher et al. 2011). Bacterial LPS has been shown to up-regulate TLR3 expression via the TLR4-MyD88-IRAK-TRAF6-NF-κB-dependent signaling pathway, for enhancing anti-viral responses (Pan, Fisher et al. 2011, Bakaysa, Potter et al. 2014). However, the role of TLR3 in bacterial infections is poorly understood and remains controversial (Thorburn, Tseng et al. 2016, He, Ichinose et al. 2017, Shirjang, Mansoori et al. 2017). The results of our study revealed the unexpected role of MyD88 in the MyD88-independent pathway for the first time, along with the role of HSMGB1 and HSP60 in the downstream activation of TLR3. This results in the generation of more inflammatory signals of TLR2 that might lead to persistent inflammation.

In a seminal paper, Lai et al. investigated a skin injury model (full-thickness incisions were performed creating a circular wound) of mice and identified the fact that

inflammation was driven by TLR3 mediated responses in keratinocytes (Perkins and Vogel 2015, Lakpour, Koruji et al. 2017, Wissler, Ehlerding et al. 2019). The results showed the necessity of TLR3 for the induction of inflammation after a skin injury. Interestingly, staphylococcal LTA-mediated TLR2 signaling suppressed TLR3 signaling via the induction of the negative regulatory factor TRAF1 (Benakanakere, Zhao et al. 2010, Granick, Falahee et al. 2013, Fang, Shi et al. 2016, Hu, Cong et al. 2016). The authors observed that RNA from necrotic epithelial cells triggered TLR3 on undamaged epithelial cells, leading to the release of pro-inflammatory cytokines, and concluded that the specificity of a ligand and its response is dictated by a cell type specific TLR2 ligand (Chalmers, Eidelman et al. 2013, Hu, Chen et al. 2018, Martinus and Goldsbury 2018). In our experiment, HGECs behaved differently than expected. TLR3 ligand stimulation in HGECs led to the synergistic activation of TLR3 and TLR2 via the induction of the endogenous ligand for TLR2. In our study when TLR3 is triggered by Poly I:C TLR2 is upregulated although in siTLR2 treated cells suggesting that TLR3 stimulation leads to a strong transcriptional activation of TLR2. Furthermore, TLR3 stimulation via Poly I:C induces endogenous ligands of TLR2 in vitro. In addition, Poly I:C increases MyD88 expression even though it is thought to be independent. Along with it, TNF and IL8 secretion were significantly downregulated upon Poly I:C treatment in MyD88 deficient mouse.

Taken together, the above data suggest that TLR2-TLR3 regulatory networks are complex and reveal their association with the TLR3-MyD88-TLR2 signaling axis. Interestingly, viral infections occurred in fetal membranes that secreted MIP-1β and

RATES (Bakaysa, Potter et al. 2014), in response to Poly I:C, via MyD88 signaling (Rajalakshmy, Malathi et al. 2015, Rashidi, Mirahmadian et al. 2015, Muralidharan, Lim et al. 2018). This suggests their association with TLR3-MyD88 signaling, which probably depends on the cell type specificity and stimulus. Nonetheless, there are no reports suggesting the involvement and cooperation of MyD88 in TLR3 signaling pathways in oral keratinocytes. The results of our study have defined the role of Myd88 in the Mydd88-independent signaling pathway for the first time. In this pathway, TLR3 activated TLR2 expression via a Myd88-dependent mechanism, and TLR3 mediated the up-regulation of endogenous ligands for TLR2, mainly, HMGB1 and Hsp60. This exacerbated TLR2 signaling during pro-inflammatory cytokine production. We believe that this discovery regarding the co-operation among TLRs for the activation of pro-inflammatory cytokines is novel; it might result in persistent inflammation if a viral infection has occurred. On the contrary, studies have provided evidence regarding the protection provided by the TLR3 and MyD88-dependent signaling pathways against viral infections. The increased susceptibility and high mortality of viral infections has been noted in $MyD88^{-/-}$.$TLR3^{-/-}$ knockout mice at the early stage of infection (Xia, Winkelmann et al. 2013, Hu, Cong et al. 2016). These data corroborate a comprehensive examination of the roles of these pathways interacting with each other during the development of long-term adaptive immune responses to viruses.

The impact of the lack of TLR3 or MyD88-dependent signaling is also manifested during B cell memory development (Xia, Winkelmann et al. 2013). When MyD88 knockout mice were treated with RepliVAX WN to determine the response of B cells, a significant

reduction in B cell activation was observed. This study also noted that the activation of TLR2 occurs via TLR3 in a downstream direction, through HSP60 (Xia, Winkelmann et al. 2013).

Human oral cavity (mouth) hosts a complex microbiome consisting of bacteria, archaea, protozoa, fungi and viruses. These bacteria are responsible for two common diseases of the human mouth including periodontal (gum) and dental caries (tooth decay) (Mosaddad, SA at al. 2019).

The focus has traditionally been on bacteria and fungi when discussing microbiological aspects of oral disease. Viruses are probably more involved in diseases associated with the oral cavity than has been previously thought. The role of several viruses in ulceration is well known, but viruses of the herpes family may play a role in periodontitis, and papillomaviruses are probably involved in oral cancer (Grinde, Bjorn at al. 2010).

It should be noted that the microbial activity can also induce viral replication, as has been shown recently in the case of EBV and malaria. If the impact of viral replication on the bacterial environment is real, then it might be expected that the bacterial profiles would differ between sites with or without virus. Such correlations have been previously reported. (Siqueira, JF at. Al 2017).

Epithelial cells in the mucosa serve as a barrier for those micro-organisms and recognize them through pathogen recognition receptors (PRRs) that instigate antibacterial and antiviral responses. The mechanism of co-operation between TLRs may help explain the

complexity of viral and bacterial infections. Poly I:C can potentially be used as an adjuvant in the development of vaccines and therapeutic approaches to enhance host immunity and reduce destructive inflammation.

Conclusion:

The results of the current study suggest that both TLR3- and MyD88-dependent signaling play important roles in shaping the development of innate immune responses. This might help to explain the occurrence of complications such as bacterial and viral infections in patients with chronic oral inflammation. However, we believe that the use of Poly I:C as an adjuvant is an excellent choice for the development of vaccines against chronic oral infections such as periodontitis, to mount a robust immune response if necessary. Furthermore, dsRNA potentially represents an additional reservoir of genetic information in microbial populations (Mindich et al. 2006). A potential source of genetic material in microbial populations is dsRNA. dsRNA is used as genomic material by some viruses that infect bacterial (Mindich et al. 2006) and eukaryotic microbial hosts. Further studies are needed to understand the synergistic activation between a bacterial receptor (TLR2) and the TLR3-and MyD88-dependent signaling mechanism.

Toll-like receptors in Orthodontics:

Fibroblasts serve as an important mechano-sensor function in the PDL and Gingiva. Fibroblasts are cells that also express TLR3 receptors. The concept of aseptic inflammation was recently strengthened by discovery of the DAMPs system, where

endogenous molecules are able to trigger inflammatory response by cellular stress or damage through the biding of toll-like receptors (TLRs) and nod-like receptors (NLRs) (Masaru, Yamagushi et al. 2015). Orthodontic tooth movement is known to cause inflammatory reactions in the periodontium and dental pulp, which will stimulate release of various biochemical mediators (Masaru, Yamagushi et al. 2015). The effect of toll like receptors stimulation on orthodontic tooth movement is still unknown and a study to investigate if the activation of TLRs affect the orthodontic tooth movement would be of a great value.

Acknowledgments:

This work was partially supported by United States Public Health Service, National Institutes of Health, and the NIDCR grant DE017384. MRB received internal funding from the School of Dental Medicine, University of Pennsylvania. We wish to express our immense gratitude towards Dr. Dana T. Graves, who helped to design key experiments and conduct critical discussions regarding the manuscript.

REFERENCES

Akira, S. and K. Takeda (2004). "Toll-like receptor signalling." Nat Rev Immunol **4**(7): 499-511.

Akira, S., S. Uematsu and O. Takeuchi (2006). "Pathogen recognition and innate immunity." Cell **124**(4): 783-801.

Akira, S., M. Yamamoto and K. Takeda (2003). "Role of adapters in Toll-like receptor signalling." Biochem Soc Trans **31**(Pt 3): 637-642.

Al-Ofi, E. A. and B. S. Al-Ghamdi (2018). "High-Mobility Group Box 1 (HMGB1), an Endogenous Ligand of Toll-Like Receptors 2 and 4, Induces Astroglial Inflammation via NF-beta Pathway." Folia Morphol (Warsz).

Alkanani, A. K., N. Hara, E. Lien, D. Ir, C. V. Kotter, C. E. Robertson, B. D. Wagner, D. N. Frank and D. Zipris (2014). "Induction of diabetes in the RIP-B7.1 mouse model is critically dependent on TLR3 and MyD88 pathways and is associated with alterations in the intestinal microbiome." Diabetes **63**(2): 619-631.

Bagchi, A., E. A. Herrup, H. S. Warren, J. Trigilio, H. S. Shin, C. Valentine and J. Hellman (2007). "MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists." J Immunol **178**(2): 1164-1171.

Bakaysa, S. L., J. A. Potter, M. Hoang, C. S. Han, S. Guller, E. R. Norwitz and V. M. Abrahams (2014). "Single- and double-stranded viral RNA generate distinct cytokine and antiviral responses in human fetal membranes." Mol Hum Reprod **20**(7): 701-708.

Barton, G. M. and R. Medzhitov (2002). "Control of adaptive immune responses by Toll-like receptors." Curr Opin Immunol **14**(3): 380-383.

Barton, G. M. and R. Medzhitov (2002). "Toll-like receptors and their ligands." Curr Top Microbiol Immunol **270**: 81-92.

Benakanakere, M. R., Q. Li, M. A. Eskan, A. V. Singh, J. Zhao, J. C. Galicia, P. Stathopoulou, T. B. Knudsen and D. F. Kinane (2009). "Modulation of TLR2 protein expression by miR-105 in human oral keratinocytes." J Biol Chem **284**(34): 23107-23115.

Benakanakere, M. R., J. Zhao, J. C. Galicia, M. Martin and D. F. Kinane (2010). "Sphingosine kinase-1 is required for toll mediated beta-defensin 2 induction in human oral keratinocytes." PLoS One **5**(7): e11512.

Blednov, Y. A., M. Black, J. M. Benavidez, A. Da Costa, J. Mayfield and R. A. Harris (2017). "Sedative and Motor Incoordination Effects of Ethanol in Mice Lacking CD14, TLR2, TLR4, or MyD88." Alcohol Clin Exp Res **41**(3): 531-540.

Blednov, Y. A., M. Black, J. Chernis, A. Da Costa, J. Mayfield and R. A. Harris (2017). "Ethanol Consumption in Mice Lacking CD14, TLR2, TLR4, or MyD88." Alcohol Clin Exp Res **41**(3): 516-530.

Brown, J., H. Wang, G. N. Hajishengallis and M. Martin (2011). "TLR-signaling networks: an integration of adaptor molecules, kinases, and cross-talk." J Dent Res **90**(4): 417-427.

Burns, E., G. Bachrach, L. Shapira and G. Nussbaum (2006). "Cutting Edge: TLR2 is required for the innate response to Porphyromonas gingivalis: activation leads to bacterial persistence and TLR2 deficiency attenuates induced alveolar bone resorption." J Immunol **177**(12): 8296-8300.

Chalmers, S. A., A. S. Eidelman, J. C. Ewer, J. M. Ricca, A. Serrano, K. C. Tucker, C. M. Vail and R. A. Kurt (2013). "A role for HMGB1, HSP60 and Myd88 in growth of murine mammary carcinoma in vitro." Cell Immunol **282**(2): 136-145.

Chen, D., W. Xie, Y. Lu, S. Su, L. Nong, Y. Jia, Y. Liu, W. Zhou, H. Wang and A. Tan (2017). "Gene polymorphisms of TLR2 and TLR3 in HBV clearance and HBV-related hepatocellular carcinoma in a Chinese male population." Int J Biol Markers **32**(2): e195-e201.

Ding, L. H., D. Liu, M. Xu, M. Wu, H. Liu, R. N. Tang, K. L. Ma, P. S. Chen and B. C. Liu (2015). "TLR2-MyD88-NF-kappaB pathway is involved in tubulointerstitial inflammation caused by proteinuria." Int J Biochem Cell Biol **69**: 114-120.

Du, K., J. Liu, R. Broering, X. Zhang, D. Yang, U. Dittmer and M. Lu (2018). "Recent advances in the discovery and development of TLR ligands as novel therapeutics for chronic HBV and HIV infections." Expert Opin Drug Discov **13**(7): 661-670.

Fang, P., H. Y. Shi, X. M. Wu, Y. H. Zhang, Y. J. Zhong, W. J. Deng, Y. P. Zhang and M. Xie (2016). "Targeted inhibition of GATA-6 attenuates airway inflammation and remodeling by regulating caveolin-1 through TLR2/MyD88/NF-kappaB in murine model of asthma." Mol Immunol **75**: 144-150.

George, J. A., S. B. Kim, J. Y. Choi, A. M. Patil, F. M. A. Hossain, E. Uyangaa, J. Hur, S. Y. Park, J. H. Lee, K. Kim and S. K. Eo (2017). "TLR2/MyD88 pathway-dependent regulation of dendritic cells by dengue virus promotes antibody-dependent enhancement via Th2-biased immunity." Oncotarget **8**(62): 106050-106070.

Getz, G. S. (2005). "Thematic review series: the immune system and atherogenesis. Bridging the innate and adaptive immune systems." J Lipid Res **46**(4): 619-622.

Gonzalez-Barranco, D., M. E. Sandoval-Islas and V. M. Trujillo-Valdes (1978). "Indirect immunofluorescense reaction in cysticercosis." Arch Invest Med (Mex) **9**(1): 51-58.

Granick, J. L., P. C. Falahee, D. Dahmubed, D. L. Borjesson, L. S. Miller and S. I. Simon (2013). "Staphylococcus aureus recognition by hematopoietic stem and progenitor cells via TLR2/MyD88/PGE2 stimulates granulopoiesis in wounds." Blood **122**(10): 1770-1778.

Gray, P., A. Dunne, C. Brikos, C. A. Jefferies, S. L. Doyle and L. A. O'Neill (2006). "MyD88 adapter-like (Mal) is phosphorylated by Bruton's tyrosine kinase during TLR2 and TLR4 signal transduction." J Biol Chem **281**(15): 10489-10495.

He, M., T. Ichinose, Y. Yoshida, K. Arashidani, S. Yoshida, H. Takano, G. Sun and T. Shibamoto (2017). "Urban PM2.5 exacerbates allergic inflammation in the murine lung via a TLR2/TLR4/MyD88-signaling pathway." Sci Rep **7**(1): 11027.

Hu, Y., Z. Chen, L. Jiang, F. Chen, R. Jin and L. Cheng (2018). "Effects of oral and subcutaneous administration of HSP60 on myeloid-derived suppressor cells and atherosclerosis in ApoE-/- mice." Biochem Biophys Res Commun **498**(4): 701-706.

Hu, Y., X. Cong, L. Chen, J. Qi, X. Wu, M. Zhou, D. Yoo, F. Li, W. Sun, J. Wu, X. Zhao, Z. Chen, J. Yu, Y. Du and J. Wang (2016). "Synergy of TLR3 and 7 ligands significantly enhances function of DCs to present inactivated PRRSV antigen through TRIF/MyD88-NF-kappaB signaling pathway." Sci Rep **6**: 23977.

Jillson, O. F. (1982). "Immunology of dermatophytosis: innate and acquired immunity." Cutis **30**(1): 27-28, 32, 35.

Kawai, T. and S. Akira (2010). "The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors." Nat Immunol **11**(5): 373-384.

Kennedy, C. L., M. Najdovska, H. Tye, L. McLeod, L. Yu, A. Jarnicki, P. S. Bhathal, T. Putoczki, M. Ernst and B. J. Jenkins (2014). "Differential role of MyD88 and Mal/TIRAP in TLR2-mediated gastric tumourigenesis." Oncogene **33**(19): 2540-2546.

Kennedy, M. A. (2010). "A brief review of the basics of immunology: the innate and adaptive response." Vet Clin North Am Small Anim Pract **40**(3): 369-379.

Kenny, E. F., S. Talbot, M. Gong, D. T. Golenbock, C. E. Bryant and L. A. O'Neill (2009). "MyD88 adaptor-like is not essential for TLR2 signaling and inhibits signaling by TLR3." J Immunol **183**(6): 3642-3651.

Koticha, K. K. (1969). "Principles of Immunology-A Brief Review Part-I Basic Immunology." Indian J Dermatol Venereol **35**(4): 178-184.

Lakpour, M. R., M. Koruji, A. Shahverdi, S. Aghajanpour, M. Rajabian Naghandar, M. A. Sadighi Gilani, M. Sabbaghian and R. Aflatoonian (2017). "The Expression of TLR2 and TLR3 in Sertoli Cells of Azoospermic Patients." Cell J **19**(3): 375-385.

Latorre, E., C. Mendoza, E. Layunta, A. I. Alcalde and J. E. Mesonero (2014). "TLR2, TLR3, and TLR4 activation specifically alters the oxidative status of intestinal epithelial cells." Cell Stress Chaperones **19**(2): 289-293.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Marcelli Barge, A., A. Benajam, J. C. Poirier and G. Querne (1979). "[Image analysis application at immuno-hematology (author's transl)]." Pathol Biol (Paris) **27**(4): 235-240.

Martinus, R. D. and J. Goldsbury (2018). "Endothelial TNF-alpha induction by Hsp60 secreted from THP-1 monocytes exposed to hyperglycaemic conditions." Cell Stress Chaperones **23**(4): 519-525.

McNutt, D. R. (1974). "Immunity. I. Review of basic immunology." Rev Bras Pesqui Med Biol **7**(1): 71-80.

Melvold, R. W. and R. P. Sticca (2007). "Basic and tumor immunology: a review." Surg Oncol Clin N Am **16**(4): 711-735, vii.

Mortazavi, S. H., R. Amin, S. Alyasin, S. Kashef, M. H. Karimi, M. Babaei and V. Younesi (2015). "Down-regulation of TLR2, 3, 9 and Signaling Mediators, MyD88 and TRIF, Gene Transcript Levels in Patients with Kawasaki Disease Treated with IVIG." Iran J Allergy Asthma Immunol **14**(2): 188-197.

Muralidharan, S., A. Lim, D. Catalano and P. Mandrekar (2018). "Human Binge Alcohol Intake Inhibits TLR4-MyD88 and TLR4-TRIF Responses but Not the TLR3-TRIF Pathway: HspA1A and PP1 Play Selective Regulatory Roles." J Immunol **200**(7): 2291-2303.

Niflioglu, G. G. and B. Lebe (2014). "[Pemphigoid gestationis: light microscopic and direct immunofluorescense findings]." Turk Patoloji Derg **30**(2): 152-154.

Pan, Z. K., C. Fisher, J. D. Li, Y. Jiang, S. Huang and L. Y. Chen (2011). "Bacterial LPS up-regulated TLR3 expression is critical for antiviral response in human monocytes: evidence for negative regulation by CYLD." Int Immunol **23**(6): 357-364.

Parham, P. (2006). "Immunology: adaptable innate killers." Nature **441**(7092): 415-416.

Perkins, D. J. and S. N. Vogel (2015). "Space and time: New considerations about the relationship between Toll-like receptors (TLRs) and type I interferons (IFNs)." Cytokine **74**(2): 171-174.

Qin, Y., H. Li and J. Qiao (2016). "TLR2/MyD88/NF-kappaB signalling pathway regulates IL-8 production in porcine alveolar macrophages infected with porcine circovirus 2." J Gen Virol **97**(2): 445-452.

Rajalakshmy, A. R., J. Malathi and H. N. Madhavan (2015). "Hepatitis C Virus NS3 Mediated Microglial Inflammation via TLR2/TLR6 MyD88/NF-kappaB Pathway and Toll Like Receptor Ligand Treatment Furnished Immune Tolerance." PLoS One **10**(5): e0125419.

Rakoff-Nahoum, S. and R. Medzhitov (2009). "Toll-like receptors and cancer." Nat Rev Cancer **9**(1): 57-63.

Rashidi, N., M. Mirahmadian, M. Jeddi-Tehrani, S. Rezania, J. Ghasemi, S. Kazemnejad, E. Mirzadegan, S. Vafaei, M. Kashanian, Z. Rasoulzadeh and A. H. Zarnani (2015). "Lipopolysaccharide- and Lipoteichoic Acid-mediated Pro-inflammatory Cytokine Production and Modulation of TLR2, TLR4 and MyD88 Expression in Human Endometrial Cells." J Reprod Infertil **16**(2): 72-81.

Sahoo, B. R., M. Basu, B. Swain, M. R. Dikhit, P. Jayasankar and M. Samanta (2013). "Elucidation of novel structural scaffold in rohu TLR2 and its binding site analysis with peptidoglycan, lipoteichoic acid and zymosan ligands, and downstream MyD88 adaptor protein." Biomed Res Int **2013**: 185282.

Sakai, K., K. M. Sanders, M. R. Youssef, K. M. Yanushefski, L. Jensen, G. Yosipovitch and T. Akiyama (2016). "Mouse model of imiquimod-induced psoriatic itch." Pain **157**(11): 2536-2543.

Shirjang, S., B. Mansoori, S. Solali, M. F. Hagh and K. Shamsasenjan (2017). "Toll-like receptors as a key regulator of mesenchymal stem cell function: An up-to-date review." Cell Immunol **315**: 1-10.

Singh, B., I. Biswas, S. Bhagat, S. Surya Kumari and G. A. Khan (2016). "HMGB1 facilitates hypoxia-induced vWF upregulation through TLR2-MYD88-SP1 pathway." Eur J Immunol **46**(10): 2388-2400.

Szaleczky, E., L. Pronai, B. Molnar, L. Berczi, J. Feher and Z. Tulassay (2000). "Increased cell proliferation in chronic Helicobacter pylori positive gastritis and gastric

carcinoma--correlation between immuno-histochemistry and Tv image cytometry." Anal Cell Pathol **20**(2-3): 131-139.

Teixeira, H., J. Zhao, D. F. Kinane and M. R. Benakanakere (2019). "IFN-beta secretion is through TLR3 but not TLR4 in human gingival epithelial cells." Mol Immunol **111**: 27-31.

Thorburn, A. N., H. Y. Tseng, C. Donovan, N. G. Hansbro, A. G. Jarnicki, P. S. Foster, P. G. Gibson and P. M. Hansbro (2016). "TLR2, TLR4 AND MyD88 Mediate Allergic Airway Disease (AAD) and Streptococcus pneumoniae-Induced Suppression of AAD." PLoS One **11**(6): e0156402.

Uematsu, S. and S. Akira (2007). "Toll-like receptors and Type I interferons." J Biol Chem **282**(21): 15319-15323.

Wissler, H. L., E. B. Ehlerding, Z. Lyu, Y. Zhao, S. Zhang, A. Eshraghi, Z. Y. Buuh, J. C. McGuth, Y. Guan, J. W. Engle, S. J. Bartlett, V. A. Voelz, W. Cai and R. E. Wang (2019). "Site-Specific Immuno-PET Tracer to Image PD-L1." Mol Pharm **16**(5): 2028-2036.

Xia, J., E. R. Winkelmann, S. R. Gorder, P. W. Mason and G. N. Milligan (2013). "TLR3- and MyD88-dependent signaling differentially influences the development of West Nile virus-specific B cell responses in mice following immunization with RepliVAX WN, a single-cycle flavivirus vaccine candidate." J Virol **87**(22): 12090-12101.

Zhao, J., M. R. Benakanakere, K. B. Hosur, J. C. Galicia, M. Martin and D. F. Kinane (2010). "Mammalian target of rapamycin (mTOR) regulates TLR3 induced cytokines in human oral keratinocytes." Mol Immunol **48**(1-3): 294-304.